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Cartilagenous Tumors (Chondroma and Chondrosarcoma) in Animals

D. J. SULLIVAN, D.V.M., M.S.

Denver, Colorado

PRIMARY CARTILAGENOUS TUMORS are found infrequently in animals. Consideration of these growths, however, reveals that they occur most commonly in sheep, which are thought to be relatively free of tumors. This first became apparent in the course of routine re-examination of the material in the files of the Animal Disease Research Laboratory, Denver, Colo. Its discovery suggested that an investigation of these tumors would be a worthwhile continuation of the study of neoplasms in food-producing animals.

REVIEW OF THE LITERATURE

Little information is available on the occurrence of cartilagenous tumors in animals. This is not surprising in view of the rarity of such growths. According to Formad,[5] "chondromas may be found in all domestic animals, especially sheep, cattle, hogs, and horses, also rather commonly in dogs and domestic fowls." However, he did not mention any cases that he had studied personally. The reports that specifically indicated the species affected included 12 cases in dogs, 5 in cattle, 3 in sheep, and 2 in horses.

Banks and Bridges[1] described a 9-year-old male dog of mixed Pekingese breeding, which suffered from multiple cartilagenous exostoses of the appendicular and axial skeleton. Gay[6] described an invasive, nonmetastatic chondrosarcoma of the frontal sinus in a 5-year-old male German Shepherd Dog. Moulton[12] found three "osteochondrosarcomas" in 107 mixed tumors of the mammary glands of dogs. Mulligan[13] found six chondrosarcomas among 1,000 canine tumors. These affected the hard palate, nasal passages, mandible, ribs, and tibia. Plummer[14] described a chondrosarcoma of the scapulohumeral articulation in a dog.

The cartilagenous tumors described in cattle have been designated chondroma and chondrosarcoma. Jackson[11] reported two chondromas, one on the ribs and one on the sternum. Plummer[14] described three chondrosarcomas affecting the sternum, with metastasis to the lungs, pleura, and pericardium. These authors also contributed two equine cases, both of which were chondromas, one affecting the external ear, the other, the nasal cartilage.

Two of the 3 cases in sheep were chondromas affecting the scapular cartilage. These were described by Chrétien[2] and by Feldman.[3] Moreover, Feldman[4] has described "chondroblastoma" and reviewed the earlier literature. The remaining cartilagenous tumor in sheep was a malignant enchondroma of the thigh, described by Hock.[8] There was metastasis to the lungs in this case.

MATERIALS AND METHODS

The files of the Animal Disease Research Laboratory, Denver, Colo., were searched for examples of cartilagenous tumors in food-producing animals. Nine such tumors were found among the approximately 1,300 neoplasms present. Of these, seven were in sheep, one in a cow, and one in a horse. This material was supplemented by 31 cases from the Chicago laboratory, 13 of which were complete. Of the complete cases, 11 were from sheep and 2 from cattle. The 18 instances in which case reports alone were available represented 14 tumors in sheep, 3 in cattle, and 1 in a horse. Thus, a total of 40 cases were available for consideration: 32 in sheep, 6 in cattle, and 2 in horses. Of these, 22 were complete: 18 in sheep, 3 in cattle, and 1 in a horse.

From the Animal Disease Research Laboratory, Animal Disease and Parasite Research Division, Agricultural Research Service, USDA, Denver, Colo. The author is now with the Section of Experimental Pathology and Toxicology, Sterling-Winthrop Research Institute, Rensselaer, N.Y.

Fig. 1 (Case D 2676)—Chondrosarcoma of the sternum of a sheep. The lesion was a solid, glistening, grayish white mass. Metric scale is at top of picture.

This material was reviewed, and the age and sex incidence of animals affected with these neoplasms were recorded. The primary site of the growths was determined whenever possible and, in cases with metastasis, the location of the metastatic lesions was noted. Finally, the gross and histologic appearance of the tumors was described, and comparisons were made of the degree of malignancy indicated by gross and microscopic findings.

OBSERVATIONS

Any attempt to define the age or sex incidence of animals with neoplasms, especially sheep, is a hazardous enterprise. Since 32 of the 40 tumors in this series were found in sheep, the information found in the postmortem reports will be quoted without further comment. These records listed the sex incidence by species: sheep, 22 females and 3 males; cattle, 3 females; and horses, 2 males. In ten instances, the sex was not stated in the reports. Most of the affected animals were adults or aged individuals. Ages were given for 27 sheep. Of these, 13 were described as being aged, adult, or more than 5 years old. In the other 14 sheep, the ages were: 4 years, 5; 3 years, 1; 2 years, 2; 1 year, 4; and less than 1 year, 2. The 3 cows were described as being old, 2 years old, and 4 months old. Both horses were old animals.

Lichtenstein [10] considers one of the distinguishing characteristics of chondrosarcoma in man to be its origin from preformed cartilage. This appears to apply to cartilagenous tumors of animals, also. In this study, 11 neoplasms in sheep originated on the ribs and 8 on the sternum. Thus, the complex cartilagenous structure formed by the sternum and the costal cartilages appeared to be the most frequent site of origin. The appendicular skeleton was the next most frequent primary site of these growths. The scapular cartilage was affected four times, various articulations, four times, and the tuber coxae, once. Of the four other ovine tumors, two were in the heart, and two were represented by multiple metastatic nodules. Since the cardiac skeleton of the sheep is composed of cartilage, it is conceivable that the two heart lesions were primary. However, these cases were represented only by case reports, and it would be pointless to postulate such an origin. These four cases have been classified as metastatic chondrosarcoma, in which no primary tumor was identified. The six tumors in cattle followed the same general pattern. They were found on the ribs, on the scapular cartilage, in the lungs in two cases, on the buccal mucosa, and as multiple metastatic nodules. In horses, the lesions were found on the ribs and in the lungs.

Approximately one third of the growths had metastasized. If one excludes the heart lesions, 11 of the sheep tumors were metastatic. The most common location of the

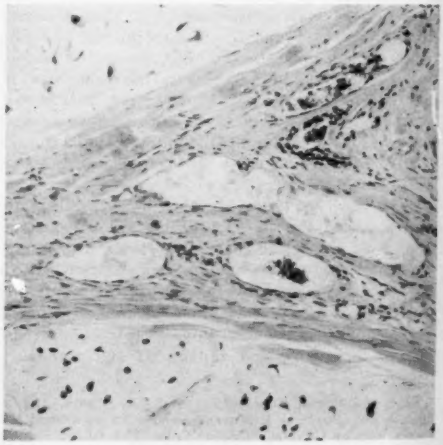

Fig. 2 (Case 17586)—Chondrosarcoma of the sternum of a sheep. Two cartilagenous loculi and the interlocular stroma are shown. The blood vessels are confined to the stroma as was typical of these tumors. H & E stain; x 110.

metastatic lesions was the lungs (10 cases). The liver and diaphragm were each affected in 5 sheep. The heart, intestine, kidney, and an unspecified lymph node were each affected once. Two of the tumors in cattle had metastasized—one to the lungs, the other to unspecified abdominal organs. One of the horses had metastatic lesions in the lungs and in the body lymph nodes.

Cartilagenous tumors in animals are fairly constant in their gross appearance (fig. 1). Usually, they are large, lobulated, glistening, bluish white structures. The size of the primary tumors in this series varied from 4 cm. in diameter to 30 by 15 by 15 cm. Metastatic lesions varied from microscopic foci to rather large structures, but they did not approach the size attained by the larger primary growths. Since these tumors are poorly vascularized, necrosis and other degenerative changes are not uncommon. Bone spicules and foci of calcification are seen frequently in the growths. Although the basic color of these neoplasms is bluish white, they may vary from white to a dark muddy red, especially if there is much necrosis present. The consistency of the lesions is also subject to variation. In general, they are firm. However, they occasionally have a gelatinous nature due to myxomatous degeneration.

The microscopic appearance of these tumors was rather uniform. The essential

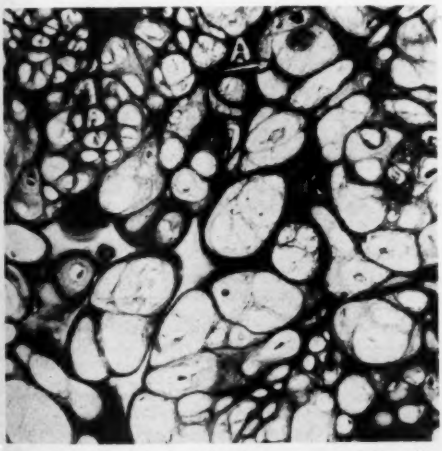

Fig. 3 (Case 16033)—Chondrosarcoma of the ribs of a sheep. This photomicrograph shows an area deep in one of the cartilagenous loculi. Marked variations in cell size are apparent. A giant cartilage cell with a plump nucleus is visible at A. Gomori's aldehyde-fuchsin stain; x 110.

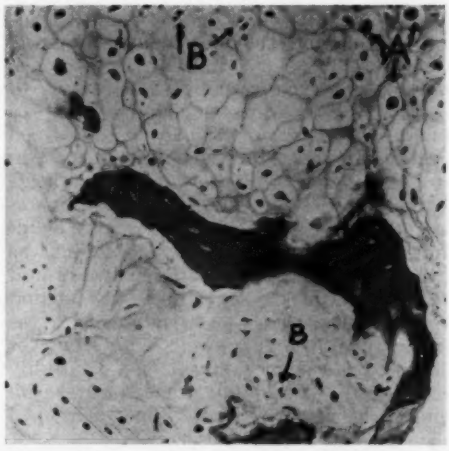

Fig. 4 (Case D 2676)—Chondrosarcoma of the sternum of a sheep. Neoplastic cells are eroding a bony lamina. There is great variation in the size of the individual cells. Cells with plump, hyperchromatic nuclei are present at A, and binucleate forms at B. H & E stain; x 110.

cell in all cases was a neoplastic chondrocyte. These cells were arranged in individual loculi of various sizes, with strands of dense connective tissue making up the interlocular stroma. The growths were poorly vascularized, with the blood vessels confined to the connective tissue stroma (fig. 2). In rare instances, the cartilagenous loculi contained blood vessels. This condition was observed only where the neoplasm was actively invading tissue, usually in the lung, and it appeared to be due to incorporation of pre-existing vessels into the tumor.

The intercellular substance of the growths was composed of fine acellular fibrils. The finest fibrils were found immediately adjacent to the neoplastic chondrocytes, and they appeared to have been produced by the chondrocytes. These fibrils coalesced to form thin interconnecting strands within the loculi, and blended with the interlocular connective tissue at the periphery of the loculus. The fibrils were stained deep blue by Gomori's aldehyde-fuchsin stain,[7] indicating the presence of mucin. With van Gieson's stain, the reaction of the interlocular connective tissue was typical of collagenous fibers.

Although the intercellular substance is an important and interesting part of any new growth, the cells are the components

on which evaluation must be based. Their appearance and behavior are the essential elements in the classification of the neoplasm. The tumors in this series were all composed of neoplastic chondrocytes. In two instances, the growths were considered benign. In these, the cells were uniform in size and shape, and both lesions had a large connective tissue component. The remaining 20 tumors, in which sections were available, were all considered malignant, as judged by the criteria of Lichtenstein and Jaffe.[11] Uniformity in cell size was lacking and, in many cases, the variations were extreme. Giant cartilage cells with single or double nuclei were common (fig. 3). Many of the cells had plump nuclei or were binucleate (fig. 4). Nuclei with clumped chromatin were encountered in five cases. Mitotic figures were seen rarely.

Cellular growth in these neoplasms did not appear to be limited to the peripheral or perichondral areas. It seemed to depend chiefly on the availability of nourishment. Growth appeared to be fairly uniform throughout the lesions unless the loculi became so large that the deeper parts were deprived of nourishment and underwent necrosis.

Neoplastic chondrocytes appear to have the ability to invade and replace bone without inciting osteoclastic activity and without causing distortion of the general shape of the body laminae (fig. 4).

DISCUSSION

Most of the tumors in this series were malignant. Of the 22 in which sections were available, only two could be considered benign.

One of the benign chondromas was found in a sheep and affected the foreleg. The other was found in the lung of a cow and appeared to have originated from bronchial cartilage. Both of these growths were single, encapsulated lesions. Histologically, they were composed of well-differentiated cartilage cells which were uniform in size and shape. In these cases, there was close correlation between the gross and microscopic findings. Both indicated a diagnosis of chondroma.

In the 20 cases that were considered to be chondrosarcoma, the correlation between gross and microscopic criteria of malignancy was not as clear cut. In 10 cases, the location or extent of the lesions found at necropsy clearly indicated malignancy, either by metastasis or by obvious invasiveness. In the 10 cases remaining, the gross lesions were single and well-circumscribed, although some of them were large. In the tumors considered to be chondrosarcoma, the cells appeared anaplastic, and there was considerable variation in size and in nuclear configuration. Mitotic figures were conspicuous in their rarity, although many binucleate forms, hyperchromatic nuclei, and nuclei with clumped chromatin were seen. These were scattered throughout the cartilagenous loculi and not confined to the perichondral areas.

SUMMARY

Forty cases of chondroma or chondrosarcoma in animals have been considered. Of these, 22 were complete with sections and were studied in detail.

Sheep were affected in 80 per cent of the cases included in this report. This, and the fact that most tumors are found in older animals, militates against attaching much significance to the preponderance of female animals in this series. Most of these tumors were found in old slaughter ewes, but a few were found in yearlings.

Of the cases studied in detail, 91 per cent were malignant as judged histologically by the standards of Lichtenstein and Jaffe.[11] Only 45 per cent were malignant as judged by commonly accepted gross criteria of metastasis or obvious invasiveness.

The most common cartilagenous tumor in food-producing animals, as indicated by examination of the material on file at the Animal Disease Research Laboratory, Denver, Colo., is chondrosarcoma in sheep. This is an uncommon neoplasm, but the fact that sheep provided 80 per cent of these tumors is noteworthy.

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SUMMARIO IN INTERLINGUA

Tumores Cartilaginose (Chondroma e Chondrosarcoma) in Animales

Le casuistica consiste de 40 casos de chondroma o chondrosarcoma in animales. In 22 de illos, le material es complete e include sectiones. Istos esseva studiate in detalio.

In 80 pro cento del casos includite in le presente reporto le animales afficite esseva oves. Iste constatation, insimul con le factor que le majoritate del tumores es trovate in animales de etates plus avantiate, suggere que pauc o nulle signification pote esser attribuite al preponderantia de femininas in le serie. Le majoritate del tumores esseva trovate in vetule oves femina de abattitorio, sed plures esseva etiam trovate in animales del prime anno.

Inter le casos studiate in detalio, 91 pro cento esseva malignitates, secundo le criterios histologic de Lichtenstein e Jaffe[11]. Solmente 45 pro cento esseva maligne secundo le communmente acceptate criterios macroscopic de metastase o obvie invasivitate.

Le plus frequente tumor cartilaginose in animales de consumo—secundo le scrutinio del documentation al Laboratorio de Recercas Zoopathologic a Denver, Colorado—es chondrosarcoma in oves. Iste neoplasma es incommun, sed le facto que oves provideva 80 pro cento del exemplos es digne de nota.

Experimental Strongyloidiasis in Sheep and Goats. IV. Migration of *Strongyloides papillosus* in Lambs and Accompanying Pathologic Changes Following Percutaneous Infection

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PREVIOUS REPORTS in this series,[22-25] were concerned with the pathogenic potentiality of experimental infections of *Strongyloides papillosus* in lambs and kids, as well as with some aspects of resistance and immunity to infection. It was demonstrated that massive, experimental infections of this parasite in otherwise parasite-free lambs and kids caused acute effects and were fatal in some cases. Although considerable information is available on the migration and development of various species of Strongyloides in tissues and organs of a number of hosts,[4, 5, 8, 13-16, 19, 30] this aspect of the biology of *S. papillosus* has been investigated previously only in small laboratory animals, particularly the rabbit,[1, 15] rather than in its natural ruminant hosts. Vegors,[27] in his study on the pathogenicity of this parasite in cattle, and Vegors and Porter,[28] in their investigation of cross-transmission of the parasite in domestic ruminants, were not concerned with the migration of the worm in these hosts. Therefore, the purpose of this paper is to report observations made on the migratory cycle of *S. papillosus* in susceptible lambs from the time the larvae penetrate the skin to the development of the adult parasites in the small intestine. The effect of larval penetration of the skin of both susceptible and resistant lambs is also described. The results of these studies in part have been previously summarized in abstract.[26]

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MATERIALS AND METHODS

Eighteen purebred Shropshire lambs were used for this work: 6 for the larval-migration studies in the skin, 4 for the purpose of making observations on the portion of the life cycle taking place in the blood stream, and 9 (1 lamb was used in preceding experiments) for the study of larvae and worms in other locations and the pathologic changes resulting from their presence. These lambs were parasite-free except for exposure to *S. papillosus*, as is indicated in the following paragraphs.

Larvae in the Skin.—Three of 6 susceptible lambs were each exposed to infection by the application of a single dose of 25,000 larvae by pipette to a selected area of the inguinal skin. Thereafter, at intervals of a quarter hour, a half hour, and 1, 12, 24, and 48 hours, biopsy specimens were taken from the area of skin exposed to larval penetration. Each of the other 3 lambs were resistant to infection after similar application of 11 separate doses of 25,000 larvae on different days over a period of two weeks. Biopsies were taken of the inguinal skin at the site of larval penetration from these immunized lambs at intervals of a quarter hour, a half hour, and 1, 12, 24, and 48 hours after the last dose of larvae was applied. Skin specimens were fixed in Bouin's fixative and stained with hematoxylin and eosin for histologic study.

Larvae in the Blood.—Four lambs were each exposed percutaneously to approximately 2,000,000 infective larvae. Three of these lambs (No. 604, 856, and 785) were anesthetized with ether, and laparotomies were performed at 12, 24, and 48 hours, respectively, after exposure to the larvae. Approximately 5 ml. of blood was removed from each of the sites listed (table 1), by means of a 10-cc. glass syringe. The lambs were then destroyed. Similar blood samples were drawn in the same manner from the jugular vein of the unanesthetized fourth lamb (No. 646) at intervals of 24, 48, 72, 96, and 120 hours after exposure to the larvae. The fresh blood was transferred to oxalated vials. Aliquots of each sample were then diluted and hemolyzed with distilled water, and the larvae were counted.

Larvae and Adults in Other Locations.—Six of the 9 lambs (No. 15, 509, 511, 515, 533, and 614) on which these observations were made were in-

fected percutaneously with 1,500,000 larvae each, and lambs 9 and 622 were infected with 300,000 and 750,000 larvae, respectively. One of these lambs was killed at each interval recorded (table 2) and was necropsied according to the following procedures. These additional observations were also made on lamb 604 (*see* previous paragraph). The peritoneal cavity was first washed gently by pipetting physiologic saline over the exposed surfaces of the viscera and abdominal wall and aspirating the washings into a 50-ml. tube. Washings from the pleural and pericardial cavities were collected in like manner from 1 lamb only (No. 509), which was killed 88 hours after infection. After this preliminary washing, each organ was removed and examined separately. Every effort was made to obtain the maximum number of larvae from weighed portions from the lungs, heart, liver, kidneys, and spleen, from measured portions of the trachea, esophagus, and spinal cord, from the contents of the abomasum, small intestine, gallbladder, and urinary bladder, and from samples of the gracilis muscle, loin, and diaphragm of 8 of these lambs. The contents of the large intestine were not examined because the finding of Strongyloides in this location during its prepatent period was considered to be most unlikely, and the time required for this procedure could be used more advantageously on other phases of the work.

Larvae were recovered from tissue samples by the incubation method described by Spindler[15] which was a slight modification of the method originally described by Fülleborn.[7] Artificial peptic digestion also was used in recovering larvae from the various organs and tissues, but it was found to be unsatisfactory because it partially destroyed the parasites. Larvae and adults of *S. papillosus* were recovered from the small intestine after tying it off at the pyloric and ileocecal valves and excising it at these points. The entire intestine was then removed and slit, the contents were washed into a suitable container, and the mucosa repeatedly rubbed manually and washed with tap water to remove any worms that might be adhering to it. The washings were preserved in 5 per cent formalin until the parasites could be enumerated. The stages of development of the worms recovered from duplicate aliquots of this material were identified and counted, and the total numbers recovered were then calculated by multiplying by the appropriate dilution factor.

RESULTS

Larvae in the Skin.—No dermatitis resulted in susceptible lambs which were exposed to single percutaneous infections. Histologic studies of the specimens obtained at biopsy showed larvae present in the skin from 15 minutes to 48 hours after exposure. Within 15 minutes after exposure, some larvae had penetrated the epidermis and were well within the corium. Larvae were also found in the sebaceous glands (fig. 1), and in various locations in the corium, but none were seen in the lymph channels although some were adjacent to them. A slight leukocytic infiltration was observed in only a few instances. This was most apparent in specimens obtained 24 hours after exposure and was characterized by leukocytes, chiefly neutrophils, which were seen in the vicinity of the invading larva (fig. 2). After 48 hours, a few larvae were seen in skin sections but appeared to have penetrated no further than those observed 15 and 30 minutes after exposure. Tissue destruction was minimal.

In lambs that received multiple exposures

TABLE I—Percutaneous Infection of Lambs with Strongyloides papillosus—Third-Stage Larvae/100 ml. of Blood Recovered During Early Prepatency

A. Anesthetized lambs (No. 604, 856, 785)

Origin of blood sample	Postinfection bleeding time (hr.); larvae recovered (No.)		
	12 hr. (604)	24 hr. (856)	48 hr. (785)
	No. larvae	No. larvae	No. larvae
Veins:			
External jugular	4,000	2,400	33
Mesenteric	1,000	20	0
Anterior vena cava	111	0	....
Hepatic-portal	3,028	200	33
Arteries:			
Carotid	.....	0	33
Aorta	56	0	0

B. Unanesthetized lamb (No. 646)

Origin of blood sample	Postinfection bleeding time (hr.); larvae recovered (No.)				
	24 hr.	48 hr.	72 hr.	96 hr.	120 hr.
	No. larvae	No. larvae	No. larvae	No. larvae	No. larvae
External jugular vein only	2,500	650	100	0	0

Absence of larvae number indicates no sample was taken.

TABLE 2—Larvae and Adults of Strongyloides papillosus Recovered from Lambs Killed at Stated Intervals After Single Percutaneous Infection

Lamb No.:	604	622	614	515	509	533	511	15	9
Postinfection killing time (hr.)	12	24	48	68	88	113	136	216	360
Tissue or organ:									
Gracilis muscle	600	246	3,598	.....	2,420	600	780	0	0
Loin	500	0	180	.....	71	55	0	0	0
Diaphragm	0	0	0	.....	150	72	0	34	0
Lungs	170	0	24	249	7,321	17,820	12,000	10	0
Trachea-esophagus	0	0	0	150	600	3,000	250	0	0
Abdominal cavity	0	0	0	0	5	0	0	0	.....
Pleural cavity	.....	.....	.....	.....	10	.....	.....	.....	.....
Pericardial sac	.....	.....	.....	.....	1	.....	.....	.....	.....
Abomasum (contents)	.....	.....	.....	0	4,290	4,000	5,000	50	100
Small intestine (contents)	.....	.....	.....	0	6,375	13,020	117,800	217,600	79,918
Totals/lamb	1,270	246	3,802	399	21,243	38,567	135,830	217,694	80,018

Larvae from muscles and lungs are given as numbers per 100 Gm.; larvae from tracheal and esophageal washings are in numbers per 4- to 6-inch sections; worms from abomasum and small intestine are totals from contents and mucosa. Absence of larvae number indicates no sample was taken.

to larvae, a slight erythema was observed shortly after the fourth larval exposure, and a pustular, erythematous dermatitis developed after the fifth and subsequent exposures. Histologic studies of the skin sections disclosed no marked changes in the lesions which could be related to the length of the interval between the last exposure to larvae and the taking of the specimen beyond one hour. The following observations were, therefore, limited to specimens removed within one hour and are considered to be typical of those seen in specimens removed at intervals of 12, 24, and 48 hours after the last exposure to larvae.

Skin sections removed after the eleventh exposure disclosed active phagocytosis of the larvae. The attendant inflammatory reaction consisted of a fluid exudate and infiltration of the tissue with eosinophils, polymorphonuclear cells, lymphocytes, and giant cells (fig. 3). Practically every larva seen in these specimens was surrounded by these cells. Many of the small veins were filled with leukocytes and giant cells. The reaction was most intense in the superficial dermis, but there were scattered cellular foci in the deep dermis adjacent to the subcutaneous tissue. In one case, 30 minutes after the final larval exposure, an area of the superficial dermis about 1 mm. in diameter was desquamated. Of the three biopsies taken, the one removed an hour after larval exposure showed the greatest polymorphonuclear response. These cells formed a micropustule in one part of the epidermis and, in the subepithelium of other areas, they were part of an acute inflammatory edema that had replaced all but a few layers of the epithelium (fig. 4). Included in the inflamed edematous areas were numerous fragments and cross sections of larvae which were not directly related to any obvious hair shafts or sebaceous glands. However, a careful search of these areas did not reveal any parasites penetrating the stratum corneum or other layers of the epithelium. Hair shafts in other parts of the section, however, contained migrating larvae accompanied by an intense cellular response (fig 3). Also, the bases of two hair shafts had been almost entirely replaced by large foci of eosinophils, but larval fragments were not demonstrated. Apparently, there was no local epidermal immunity because numerous parasites were present in the dermis, but the intense inflammatory reaction in the dermis was destroying most, if not all, of the larvae observed therein.

Larvae in the Blood.—Larvae were found in the circulating blood from 12 to 72 hours after infection (table 1). More larvae were recovered from blood 12 hours after infection than at subsequent intervals. Also, at this time, more larvae were recovered from blood obtained from the jugular vein than from that of the other vessels.

The number of the larvae in the blood stream 24 hours postinfection was usually markedly decreased as compared with that

recovered at 12 hours, but the number recovered from the jugular vein remained relatively high. Except for a relatively large number of larvae in the jugular vein, only small numbers were recovered from the other vessels 48 hours after infection. At 72 hours postinfection, a few larvae were still recovered from the jugular vein of the unanesthetized lamb, but none were found at 96 and 120 hours postinfection. Blood from the other sites was not examined at this time. All larvae recovered from the circulatory system were in the third stage of development.

Larvae and Adults in Other Locations.—No intestinal threadworms were recovered from the heart, liver, kidney, spleen, gallbladder, urinary bladder, and spinal cord of the 8 lambs in which these organs were examined. However, worms were encountered in the locations recorded (table 2). The data (table 2) show that the gracilis muscle, loin, and lungs were invaded by larvae as early as 12 hours after infection. The failure to recover larvae from the loin and lungs of lamb 622 at 24 hours after infection may be explained by the much smaller dose of larvae which was administered to this lamb as compared with that given to the other lambs that yielded larvae from these locations. Forty-eight hours after infection, many larvae were present in the gracilis muscle, and small numbers were found in the loin and lungs, but none were recovered from the trachea and esophagus. At 68 hours after infection, larvae were first recovered from the trachea and esophagus. Larvae were first detected in the abomasum, small intestine, diaphragm, and abdominal, pleural, and pericardial cavities 88 hours after infection, and they were still present in the other locations where they had previously been observed.

The number of larvae in the lungs attained its maximum 113 hours after infection and appeared to be positively correlated with the increase in the number of larvae in the trachea, esophagus, and small intestine. The relative constancy of the number of larvae in the abomasum from 88 to 136 hours postinfection appeared to indicate that a steady migration of larvae from the lungs to the small intestine was taking place during this 48-hour period. It is also significant that, after 113 hours postinfection, the number of larvae increased in the small intestine as the number decreased in the lungs. Nine days (216 hours) after infection, a few larvae were still recovered from the lungs but, at 15 days (360 hours) postinfection, the worms were found only in the abomasum and small intestine.

Although these data demonstrate that larval migration does occur between the lungs and small intestine, it must be recognized that possibly not all of the larvae present in the lung tissue were recovered because of the methods used. It is likely, therefore, that many more larvae were actually present in the lamb than were recovered, and that a larger proportion of the total larvae present were recovered from the intestinal contents than from the lungs.

Development of S. papillosus.—Larvae recovered from the lungs up to and including those observed 48 hours after infection were in the third stage. At 68 hours postinfection, 1 early fourth-stage larva was recovered from the lungs, but most of the larvae were still in the third stage. Up to and including 136 hours after infection, no larvae beyond the fourth stage were recovered from the lungs and alimentary tract; only third-stage larvae were recovered from other locations. Gravid adults were first noticed in the small intestine nine days (360 hours) after infection. These were accompanied by both third- and fourth-stage larvae as well as by young adults. More than 90 per cent of the worms in the intestine were mature 15 days after infection, and the others were in the late fourth stage.

Pathologic Changes.—The first gross pathologic changes were observed in the lungs of the lamb that was killed 48 hours after infection (table 2). A slight hemorrhage and some edema in the upper portions of the apical and diaphragmatic lobes were noticed. Sixty-eight hours after infection, petechial hemorrhages were concentrated in the upper portion of the left lung. At 88 hours postinfection, many subpleural, petechial, and ecchymotic hemorrhages had appeared over the surfaces of both lungs, and more hemorrhages were seen in the anterior portion of the diaphragmatic lobes than in the posterior portion. Maximum hemorrhage occurred in the lungs of the lamb that was killed 113 hours after infection. The lesions in the lungs were not as

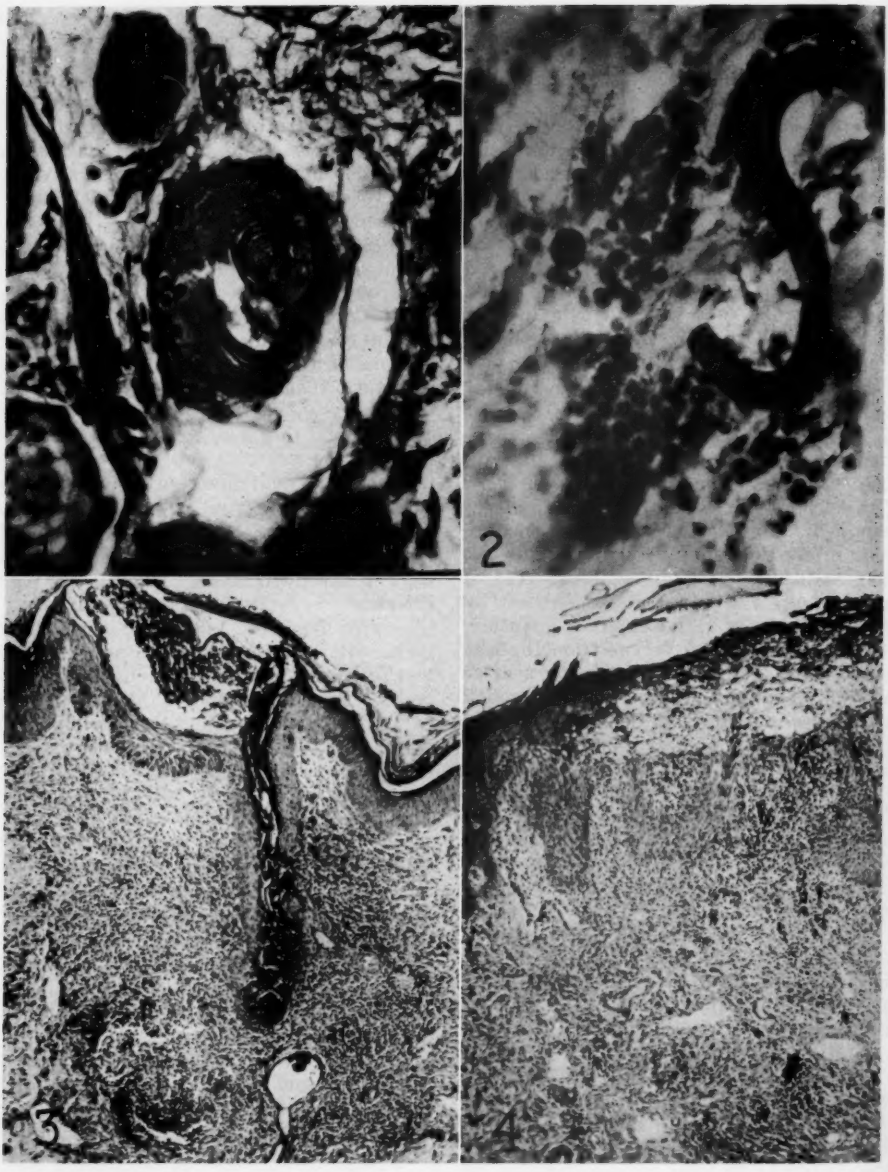

Plate I

Fig. 1—Larva of Strongyloides papillosus in sebaceous gland of lamb 30 minutes after single cutaneous infection. x 160.

Fig. 2—Skin, 24 hours after single larval exposure, showing a slight inflammatory reaction around a larva. In addition to the polymorphonuclear leukocytes, two macrophages and a fibroblast are present. x 320.

Fig. 3—Skin, 15 minutes after the eleventh larval exposure. An intense polymorphonuclear response is in the vicinity of a hair follicle. x 28.

Fig. 4—Skin, one hour after the eleventh larval exposure, illustrating the formation of a micropustule in the epidermis, as well as diffuse dermatitis with the small veins filled with leukocytes. x 28.

marked at 136 hours as at 113 hours after infection, but petechiae and ecchymoses were present over most of the lung surfaces (fig. 9, 10). Only scattered petechiae were present in the lungs of the lamb killed nine days after infection. The lungs appeared normal in the lamb that was necropsied 15 days after infection.

Histologic sections from the lungs of the lamb killed 113 hours after infection revealed acute hyperemia with much extravasation of blood in the interstitial tissue and into the alveoli (fig. 5, 6).

In these experiments, no severe damage was noticed in the small intestine of the lambs which were killed during the first 15 days of the infection. However, severe damage to intestinal tissues was previously reported [23] as early as the thirteenth day after experimental infection with 500,000 larvae. The general aspects of the pathologic changes in the intestine in patent infections were given in previous reports;[22, 23] these were extensive erosion of the duodenal and jejunal mucosae, edema, and leukocytic infiltration. Some of these changes are illustrated (fig. 7, 8). These tissues were obtained from a lamb which died 18 days after exposure to 200,000 larvae and was involved in the experiment reported [23] previously. Parasites were seen in these tissue sections deep in the intestinal glands, but there was no penetration of the muscularis mucosa (fig. 8).

DISCUSSION

Migratory Cycle in the Host.—The results of these studies showed that migration of the larvae of *S. papillosus* in lambs was usually analogous to that previously reported by many investigators [5, 8, 13, 14, 16, 19, 20] for other species of this genus in other hosts.

Histopathologic studies of larvae in the skin of lambs showed that they entered the skin rapidly, penetrating into the dermis within 15 minutes after being placed on the skin surface. During their passage through the skin, these larvae either *(a)* penetrated the stratum corneum, the cellular layers of the epidermis, and the extracellular barriers presented by the basement membrane and ground substance of the connective tissue of the dermis, or *(b)* entered the host through the epithelial tissues of the sebaceous glands and hair follicles. These larvae have no obvious organellae or structures for mechanical penetration of intact skin, but they may produce secretions which facilitate penetration. Skin penetration by *S. papillosus* was probably aided by the rapid motions of the larvae themselves, in addition to the action of some enzyme or enzyme-like secretions. Oshio and Furuta [17] believed the secretion of hyaluronidase might aid the larvae of *Strongyloides ransomi* to penetrate the skin of pigs. However, Lewert and Lee [11] found that this enzyme had no effect on the skin penetration of rats by larvae of *Strongyloides ratti* and later reported [12] that skin penetration was associated with the production by the larvae of a collagenase-like, proteolytic enzyme.

The larvae of *S. papillosus,* as well as those of *S. ratti,* as reported by Lewert and Lee,[11] apparently traversed the epidermis and the underlying components perpendicularly, rather than tunneling parallel to the epidermis. They also took advantage of the sebaceous glands and the hair follicles in entering the subcutis. Oshio and Furuta [17] found the latter to be true of larvae of *S. ransomi.* Oshio [16] reported that *Strongyloides stercoralis* and *S. ransomi* utilized the lymphatics as well as the blood vessels in their migration, but no larvae of *S. papillosus* were seen in the lymph channels of the skin during the present studies. It is probable, however, that this route was used in gaining access to the circulatory system, also. The writers only examined the lymph nodes adjacent to the site of larval penetration 12 hours after infection, but sections of the skin made within an hour after larval exposure did not show larvae in the lymph channels.

Basir [2] demonstrated in rabbits the necessity for larvae of *S. papillosus* to migrate through the blood stream, because larvae injected directly into the stomach penetrated the stomach wall and entered the circulation. This point was not checked in our sheep. From our studies, it appeared that about the same number of larvae was recovered per unit quantity of blood from the external jugular vein as was recovered from twice the quantity of blood from the hepatic-portal and mesenteric veins 12 hours after infection. This indicated that all the larvae were not removed from the circulation during their first passage through the lungs,

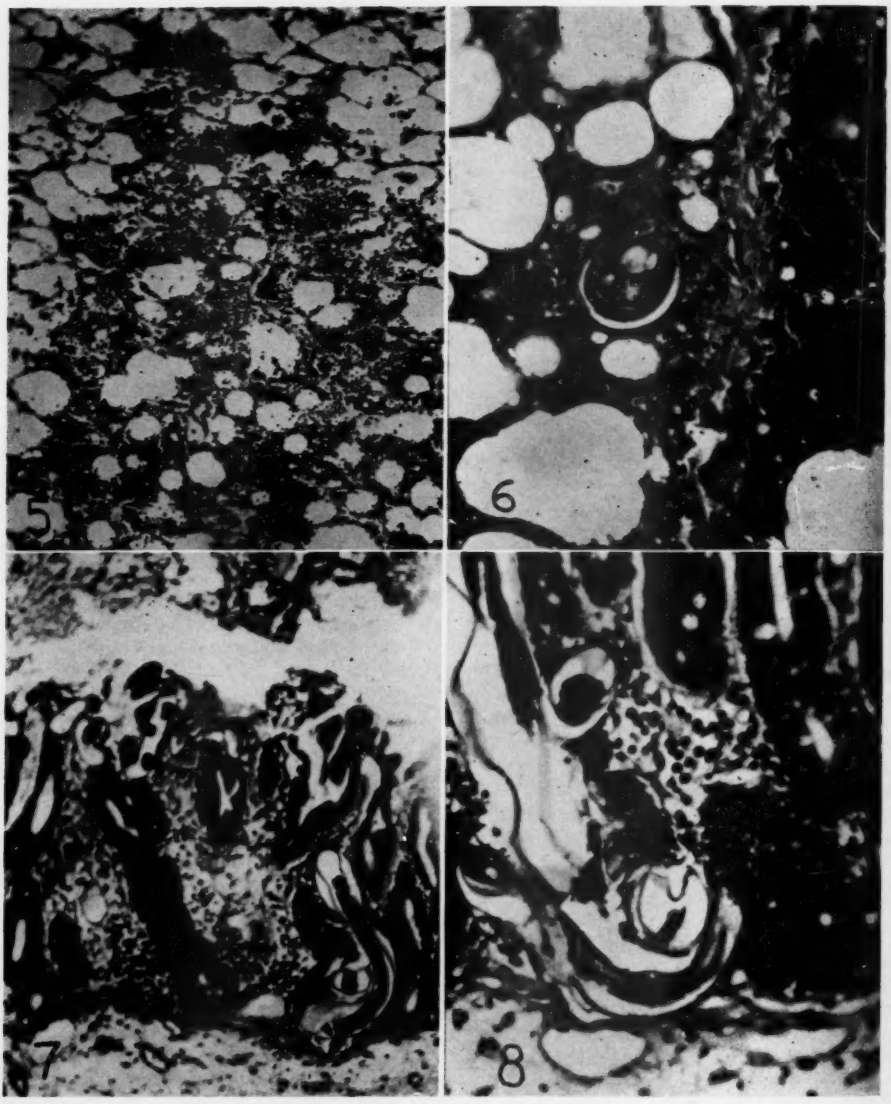

Plate 2

Fig. 5—Lung of lamb 113 hours after infection with Strongyloides papillosus, shows marked hemorrhage into the interstitial tissue and into the alveoli. x 70.

Fig. 6—Lung, 113 hours after infection, shows hemorrhage with some polymorphonuclear leukocytic exudate adjacent to a larva. x 145.

Fig. 7—Intestinal mucosa, 18 days after infection. Parasites at the base of the intestinal gland and near mucosal surface. There is partial destruction of the mucosal epithelium with polymorphonuclear leukocytic infiltration. x 70.

Fig. 8—Intestinal mucosa, 18 days after infection, showing a parasite that has penetrated to the muscularis mucosa. x 145.

XUM

but many larvae migrated from the arterial to the venous system within the lungs, returned to the heart, and then recirculated throughout the body. Therefore, the large number of larvae in the external jugular vein 12 hours after infection probably arrived at this site via the capillary bed of the head and neck regions. The finding of significant numbers of larvae in the mesenteric and hepatic-portal veins at this time was expected because these veins drain the general region of larval application. By 24 hours after infection, there was a marked decrease in the numbers of larvae in the blood, and by 48 hours, only a very few remained. Many larvae were probably delayed in, or prevented from, reaching the lungs by temporarily or permanently being held in the skin and other organs and tissues of the body.

Some larvae reached the lungs 12 hours after infection, but they were not recovered from the lungs in large numbers until 88 hours postinfection. The recovery of 1 larva from the lungs in the third molt at 68 hours confirmed Basir's [1] discovery of larvae of *S. papillosus* in the third molt in the lungs of a rabbit 32 hours after infection and supported Lucker's [13] postulation that larvae of *S. ransomi* may undergo the third ecdysis in the lungs of pigs. However, because the writers recovered parasitic larvae of *S. papillosus* in all stages of development from the intestines of lambs, the molting in the lungs does not appear essential in their development. Fülleborn [8] believed that the lung phase in its entirety was unnecessary in the normal life cycle of *S. stercoralis* in dogs, but this does not appear to be true for *S. papillosus* in sheep.

In the lungs, the larvae of *S. papillosus* migrated from the small capillaries into the alveoli and, subsequently, were carried up the trachea and into the esophagus. Many larvae first arrived in the stomach and small intestine slightly less than four days after infection. This is in close agreement with the results of Basir [1] who reported that larvae of this parasite were found in the intestine of rabbits three days after infection. He also reported that the larvae made rapid growth in the intestine of rabbits, and the final molt occurred six days after infection. Therefore, because we recovered the first adult parasites nine days after infection in lambs, the parasitic cycle of *S. papillosus* in sheep apparently requires a day or two longer than in the rabbit. The writers observed that, during the sixth day of infection, the number of larvae increased in the intestine of the lambs and decreased in the lungs, indicating that the majority were probably on their way to the small

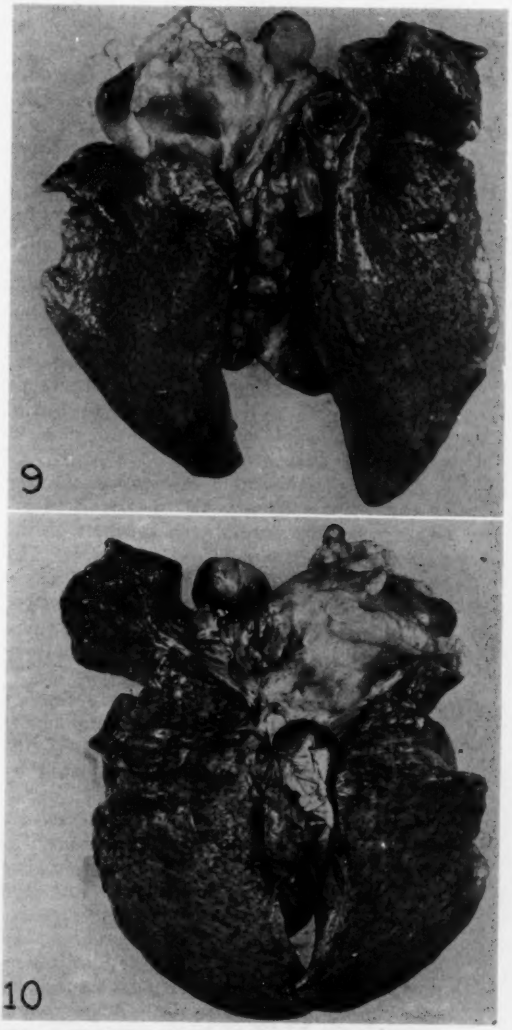

Fig. 9—Ventral surface of lungs of lamb 136 hours after infection with Strongyloides papillosus, showing hemorrhages typical of the early stages of strongyloidiasis.

Fig. 10—Dorsal surface of lungs illustrated in figure 9.

intestine at this time. Larvae were recovered up to five and two-thirds days from the gracilis muscle adjacent to the site of inoculation; thus, some larvae were delayed in their migration to the intestine. No larvae were recovered from the spinal cord of these massively infected lambs. This finding does not support the suggestion of Watanabe and Nakamura [29] that *S. papillosus* may be concerned in the etiology of lumbar paralysis in sheep.

Accompanying Pathologic Changes.—It was apparent from the writers' histopathologic observations that larvae of *S. papillosus*, in the numbers employed, caused little reaction in the skin of susceptible lambs. This finding was similar to that of Oshio and Furuta [17] who reported that little damage was caused to the skin of pigs when first exposed to larvae of *S. ransomi*. However, Tomita [21] observed the formation of vesicles on the skin of humans after application of larvae of *S. papillosus*. Man, of course, is an abnormal host for this parasite, which may account for the difference in reaction of the human skin to initial penetration of infective larvae of this ruminant parasite.

An intense pustular dermatitis, similar to that reported earlier,[24] occurred in repeatedly infected lambs as a result of the fifth and subsequent larval exposures. The repeated applications of larvae on the skin provoked an intense inflammatory reaction, and almost every larva seen in sections was undergoing destruction by inflammatory cells. This local defense mechanism in the skin against repeated invasion by larvae of *S. papillosus* is likely an important aspect of the general immune response in ovine strongyloidiasis as previously reported by Turner.[24,25]

Damage to the lungs of lambs caused by the passage of larvae of *S. papillosus* was limited to the early stages of infection. The lambs necropsied nine days after infection and thereafter did not exhibit any extensive pathologic changes in these organs. Although our finding is the first detailed report of acute lung hemorrhages caused by invasion of *S. papillosus*, lung hemorrhages caused by migrating larvae of other species of Strongyloides in other hosts have been previously reported, *i.e.*, *S. ransomi* in pigs by Spindler *et al.*,[20] *S. stercoralis* in dogs by Fülleborn [6] and Koutz and Groves,[10] Strongyloides sp. in a chimpanzee by Blacklock and Adler,[3] and *S. stercoralis* in man by Magarinos Torres and Penna de Azevedo.[14] Fülleborn [7,9] suggested that larvae of Ascaris and Ancylostoma, which have a diameter greater than 0.013 and 0.02 mm., respectively, at the time they reach the lungs of their hosts, are larger than the lung capillaries and break through them. Larvae of *S. papillosus* recovered from the lungs of the experimental lambs reported here measured up to 0.02 mm. in diameter. Therefore, the severe bleeding in the lungs of these animals, as a result of larval migration through these organs, was probably caused by larvae rupturing the pulmonary capillaries. Fülleborn [9] believed that the degree of hemorrhage depended also on the structure of the tissue invaded by the larvae of Strongyloides. He stated that brain hemorrhages occurred "only in the loose tissue of the meninges, practically never in the nervous substance." Thus, in the loose tissue of the lungs, the capillaries are not as firmly supported as in the nervous tissue of the brain, and the forceful motions of the larvae "break the delicate walls separating the air vesicles."

Turner [23] reported that the normal habitat of adult *S. papillosus* was the proximal portion of the small intestine, specifically the duodenum and proximal portion of the jejunum. It was noticed, also, that pathologic changes in this organ were usually limited to later stages of infection. Larvae were found to penetrate only to the muscularis mucosa which apparently acted as a barrier to further penetration. This confirmed the finding of Enigk [5] who found that *S. ransomi* larvae in pigs did not burrow into the submucosa of the intestine.

SUMMARY

1) The normal path of larval migration of *Strongyloides papillosus* through the body of sheep after percutaneous infection was studied in 12 lambs and found to be similar to that described for certain other species of Strongyloides. They penetrated the unbroken skin and capillary walls and were carried by the circulating blood to the lungs where they again broke through the capillary walls into the air passages, migrated up the trachea and down the esophagus, and moved through the stomach to the

small intestine where they developed to maturity. The minimum elapsed time for larvae to migrate from the surface of the skin to the small intestine was three and one-half days. The minimum prepatent period was nine days.

2) Third-stage larvae were recovered from the circulating blood from 12 to 72 hours postinfection.

3) In addition to the normal path of migration, larvae were carried or wandered to other parts of the body, also. Some were recovered from the gracilis muscle, the loin, the diaphragm, and the abdominal cavity.

4) Infective larvae rapidly penetrated the skin of susceptible lambs and were found in the dermis within 15 minutes after exposure, and some were still present 48 hours after infection. In 3 susceptible lambs, mechanical destruction of skin tissue was minimal after larval penetration, nor were the larvae hindered in their migration through the skin. However, after larval exposure, the skin of 3 resistant lambs showed diffuse dermatitis and inflammation characterized by edema and a marked leukocytic infiltration of the superficial dermis as well as desquamation of the superficial epithelium. Histologic study of biopsies from these lambs showed that the larvae were undergoing phagocytosis.

5) Hemorrhages of the petechial and ecchymotic types were severe in the lungs of the infected lambs killed during the prepatent period of the infection, but these disappeared nine days after infection.

6) No severe damage was noticed in the small intestine of infected lambs concerned in this report up to the fifteenth day after infection, but severe damage was previously reported [23] as early as the thirteenth day.

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SUMMARIO IN INTERLINGUA

Strongyloidiasis Experimental in Oves e Capras
IV. Migration de Strongyloides Papillosus in Agnos e le Accompaniante Alterationes Pathologic post Infection Percutanee

1. Le circuito normal del migration larval de *Strongyloides papillosus* a transverso le corpore de oves post infection percutanee esseva studiate in 12 agnos e trovate simile a illo describite pro certe altere species de strongyloides. Le larvas penetrava le intacte pelle e pariete capillar e esseva portate per le sanguine circulante usque al pulmones ubi illos erumpeva de novo per le pariete capillar a in le vias respiratori pro migrar in alto al longo del trachea e in basso al longo del esophago, attingente assi le stomacho e postea le intestino tenue ubi illos permaneva usque al completion de lor maturitate. Le intervallo minime pro le migration del larvas ab le superficie del pelle usque al intestino tenue esseva tres dies e medie. Le minimo del periodo prepatente esseva novem dies.

2. Larvas del tertie stadio esseva retrovate in le sanguine circulante a periodos de inter 12 e 72 horas post le infection.

3. A parte le circuito normal del migration, larvas esseva portate o migrava etiam a altere partes del corpore. Illos esseva retrovate in le musculo gracile, le lumbo, le diaphragma, e le cavitate abdominal.

4. Larvas infective penetrava rapidemente in le pelle de agnos susceptibile. Illos esseva trovate al interior del derme intra 15 minutas post le exposition; certes esseva ancora presente 48 horas post le infection. In 3 susceptibile agnos, le destruction mechanic del pelle esseva minimal post le penetration del larvas le quales incontrava nulle impedimento in lor migration percutanee. Tamen, post exposition al larvas, le pelle de 3 resistente agnos monstrava diffuse dermatitis e inflammation characterisate per edema e marcate grados de infiltration leucocytic del derme superficial e etiam desquamation del epithelio superficial. Le studio histologic de biopsias ab iste agnos monstrava que le larvas suffreva phagocytosis.

5. Hemorrhagias de typo petechial e ecchymotic esseva sever in le pulmones del inficite agnos que esseva sacrificate durante le periodo prepatente del infection. Novem dies post le infection, omne tal hemorrhagias habeva disparite.

6. In le inficite agnos del presente reporto, nulle serie traumatisation esseva vidite usque al dece-quinte die post le infection, sed in un previe reporto le presentia de serie traumatisation esseva jam notate post dece-tres dies.

Serologic Relationship of New Feline Cytopathogenic Viruses

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REPORTS by Crandell and Maurer[3] and Fastier[4] on the isolation of tissue culture cytopathogenic agents from cats, as well as information by Madin and McKercher referred to in a review by Sinha,[6] suggest that this species harbors a number of viruses, some of which may be involved in the feline disease picture. An additional group of viruses has been recovered at this laboratory as a result of studies with feline respiratory diseases. Efforts to establish the antigenic relationships of these various agents are described in this report.

MATERIALS AND METHODS

Tissue Culture Procedures.—Cortical tissue of kidneys from 8- to 12-week-old kittens was trypsinized by a method similar to that described by Youngner.[7] The cells were washed, centrifuged at 600 r.p.m., and resuspended in a ratio of 1 part packed cells to 200 parts of medium consisting of 0.5 per cent lactalbumin hydrolysate and 10 per cent horse serum in Earles' basic salt solution with 200 units of penicillin and 200 μmg. of streptomycin per milliliter. This was dispensed in 1.0-ml. amounts into tubes which were allowed to stand stationary until good growth was observed. The fluid was then changed, using the same medium with serum concentration reduced to 5 per cent, and the tubes placed in roller drums.

Viruses.—Virus isolations were made by swabbing the nasal and conjunctival membranes of cats with moist, sterile cotton swabs which were then placed in 2 ml. of tissue culture medium containing antibiotics. The fluid was transferred in 0.2-ml. amounts into each of several culture tubes and incubated at 35 C. for seven days. At least two blind passages were made before any sample was discarded. When a cytopathic effect (CPE) occurred, several passages were made to demonstrate the isolation of a transmissible agent. Then a pool of infected tissue culture fluid was prepared and dispensed into ampules which were sealed and stored at −70 C. Other virus isolation attempts were made by inoculating 0.2 ml. of spleen suspension into tissue culture tubes.

Preparation of Antiserums.—For convenience of discussion only, the viruses have been divided into two groups. For 9 viruses in group 1, antiserums were prepared by inoculating 1.0 ml. of live, undiluted tissue culture virus, clarified by low-speed centrifugation, intradermally into each of 2 to 6 rabbits twice a week for three weeks. Antiserums against the viruses in group 2 were prepared by a similar method, except that the antigens were frozen and thawed three to four times before clarification, and the rabbits were inoculated both intravenously and intradermally. Pre- and postinoculation serums were stored in 1.0-ml. amounts at −20 C.

Titrations and Neutralization Tests.—Titrations were made by inoculating 0.2 ml. of virus suspension into tissue culture tubes, using four tubes for each tenfold dilution. The 50 per cent end point of infectivity ($t.c.i.d._{50}$) was calculated by the Reed-Muench method.[5] A preliminary screening neutralization test was performed for each virus by mixing dilutions of virus estimated to contain 20, 100, and 200 $t.c.i.d._{50}$, respectively, with an equal amount of antiserum in a final dilution of 1:25. The mixtures were incubated at room temperature for two hours and each inoculated into four tissue culture tubes. A simultaneous virus control titration was carried out. The cultures were incubated at 35 C. and examined for a CPE at three and seven days postinoculation. All viruses neutralized by the 1:25 dilution were retested with tenfold dilutions of the same serum to obtain dilution end points.

RESULTS

Identification of Viruses. — Employing the methods described, 17 viruses were isolated from cats showing clinical signs of rhinitis and conjunctivitis. One agent was isolated from the spleen of a cat believed to be ill with feline enteritis. Efforts to isolate CPE organisms from the upper respiratory tract and conjunctiva of 50 apparently normal cats were unsuccessful. All agents produced cytologic changes in tissue cul-

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ture, often as early as 24 hours after inoculation. The cells were usually destroyed in three to five days. Virus titers ranged from 10^5 to 10^7 t.c.i.d.$_{50}$/0.2 ml. Although some variation in these features was observed, it was not possible to differentiate the agents by physical characteristics. However, neutralization tests with 12 of the viruses isolated at this laboratory and 11 furnished by other investigators indicated that these viruses could be conveniently divided into two groups, as summarized (tables 1, 2). Some viruses in group 1 showed a low serologic cross-relationship. However, when a formula proposed by Archetti and Horsfall [2] was applied, 9 of these viruses proved to be separate prototypes. Three of the last 6 viruses (table 1) showed low neutralization titers with the test serums, but complete identification cannot be made until homologous antiserums are prepared.

By contrast, all viruses in group 2 (table 2) were shown to be closely related antigenically. The agents in this group produced low antibody titers and, in fact, only two strains (F-18 and F-16) produced usable titers by hyperimmunizing techniques. Repeated attempts with C-27 virus (Crandell) failed to produce more than transient antibody titers in rabbits.

Attempts at Propagation in Other Mediums.—In an effort to identify these viruses more completely, all isolates were inoculated in 0.5-ml. amounts via the yolk sac into groups of 6 or more 7-day embryonating chicken eggs and incubated at 35 C. for nine to 12 days. No deaths occurred among the embryos. Smears of yolk sac

TABLE 1—Cross-Neutralization Studies with Group 1 Feline Cytopathogenic Viruses

Virus	t.c.i.d.$_{50}$	Type of antiserum: KCD	FR	FS	FI	FC	FJ	FPL	F-17	C-14
KCD	1,000	1:1,000	1:20	.....*	.....	.....	1:5	.....	N.T.	.....
	500	1:2,000	1:20	.....	.....	.....	1:320	.....	N.T.	.....
	100	1:5,000	1:32	.....	.....	1:32	1:320	1:10	1:5	1:32
FR	200	.....	1:1,000	.....	.....	.....	1:20	1:5	.....	.....
	100	1:10	1:3,200	1:5	.....	1:32	1:50	1:20	.....	.....
	20	1:200	1:10,000	1:32	1:10	1:200	1:2,000	1:32	1:10	1:5
FS	100	.....	.....	1:200	.....	.....	.....	.....	.....	.....
	50	.....	.....	1:200	.....	.....	.....	1:20	.....	.....
	10	.....	1:5	1:320	N.T.	.....	.....	1:20	N.T.	N.T.
FI	1,500	.....	.....	.....	N.T.	.....	.....	.....	.....	.....
	300	.....	.....	.....	1:320	.....	.....	.....	.....	.....
	30	1:10	.....	.....	1:500	.....	.....	.....	1:20	.....
FC	100	1:20	1:200	.....	.....	1:500	1:10	1:5	.....	.....
	50	1:50	1:320	1:20	.....	1:1,000	1:50	1:32	1:10	1:10
	10	1:200	1:320	N.T.	N.T.	1:5,000	1:200	1:50	N.T.	N.T.
FJ	640	.....	.....	N.T.	N.T.	.....	1:2,000	.....	N.T.	N.T.
	320	1:10	1:20	.....	.....	.....	1:2,000	.....	1:32	1:20
	64	1:50	1:50	1:10	1:10	.....	1:20,000	.....	1:100	1:32
FPL	200	.....	.....	.....	.....	.....	.....	N.T.	.....	.....
	100	.....	.....	.....	.....	.....	.....	1:10,000	.....	.....
	20	.....	.....	1:32	.....	.....	.....	N.T.	.....	.....
F-17	200	.....	.....	.....	.....	.....	.....	.....	1:500	.....
	100	.....	.....	.....	.....	.....	.....	.....	1:500	.....
	20	1:32	.....	1:20	.....	1:5	.....	.....	1:1,000	.....
C-14	200	.....	.....	1:10	.....	.....	.....	.....	.....	1:200
	100	1:32	.....	1:32	.....	.....	.....	.....	1:10	1:500
	20	1:50	.....	1:50	.....	.....	.....	.....	1:32	1:1,000
F-5	50	1:100	.....	1:50	.....	.....	.....	.....	1:20	.....
	10	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
F-9	1,000	N.T.	.....	N.T.	N.T.	.....	.....	.....	N.T.	.....
	500	N.T.	.....	N.T.	N.T.	.....	.....	.....	N.T.	.....
	100	1:20	.....	1:32	.....	.....	.....	.....	1:10	.....
C-29	64	.....	.....	.....	.....	1:10	.....	.....	.....	.....
	32	1:20	.....	.....	.....	1:10	.....	1:20	.....	.....
F-19	50	.....	.....	.....	.....	.....	.....	.....	.....	.....
F-10	50	.....	.....	.....	.....	.....	.....	.....	.....	.....
F-11	50	.....	.....	.....	.....	.....	.....	.....	.....	.....

* ..... Indicates no neutralization. N.T. = No test. The following viruses in group 2 showed no neutralization with the above antiserums: C-27, F-18, F-16, F-1, F-2, NIH, F-14, and PV.

XUM

cells, stained by Machiavello's method, failed to reveal the elementary bodies ordinarily associated with feline pneumonitis virus. In addition, each of the viruses was placed in PPLO medium, according to a method described by Adler [1] for poultry PPLO organisms. No demonstrable growth occurred. Inoculations of infected tissue fluid into other laboratory mediums, such as thioglycollate broth and tryptose agar, also failed to produce growth.

Cytologic Changes. — For viruses in group 1, the cells began to swell throughout the cell sheet within 24 hours after inoculation. They then became granular, decreased in size either singly or in clusters, and fell off the wall of the tube. A complete effect was obtained within three to four days. Viruses in group 2, while affecting individual cells in a similar manner, initially produced cytologic changes at the periphery of the cell sheet, and two to three more days were required to destroy all the cells. Stains of tissue cultures infected with group 2 viruses showed that all produced intranuclear inclusions during the later stages of growth, as described by Crandell for C-27.

TABLE 2—Cross-Neutralization Studies with Group 2 Feline Cytopathogenic Viruses

Virus	t.c.i.d.$_{50}$	Antiserum titer F-18	Antiserum titer F-16
C-27	100	1:10	1:100
	50	1:100	1:100
	10	1:500	1:1,000
F-18	400	1:32	1:200
	200	1:32	1:200
	40	1:100	1:1,000
F-16	2,000	1:32	1:100
	1,000	1:50	1:100
	200	1:100	1:320
F-1	6,300	1:10	1:32
	3,150	1:20	1:320
	630	1:32	1:320
F-2	1,250	1:10	1:320
	625	1:32	1:320
	125	1:32	1:500
PV	120	1:32	1:320
	60	1:32	1:500
	12	1:32	1:1,000
NIH	400	1:20	1:200
	200	1:32	1:200
	40	1:32	1:500
F-14	2,000	1:10	1:32
	1,000	1:32	1:100
	200	1:32	1:320

DISCUSSION

The isolation of a series of antigenically different viruses from the feline upper respiratory tract and conjunctiva indicates that this species is capable of being infected by a number of viruses. The fact that all the isolates included in this study, except three (KCD, F-20, and FPL), were obtained from cats showing clinical signs of upper respiratory disease, and that attempts to isolate CPE-producing agents from apparently normal cats were unsuccessful, strongly suggests that each of these viruses has a disease-producing potential. A preliminary serologic field study showed that neutralizing antibodies for several of the viruses of group 1 were present in 40 to 50 per cent of young kittens tested, while the incidence rose to 70 to 90 per cent for adult cats. This suggests widespread infection by at least some of these agents. Although viruses in group 1 appeared to be serologically separate entities, nevertheless there was a low titer cross-relationship between a number of them. Thus, it might be possible that a vaccine containing one or two of these would afford sufficient cross-immunity to protect the cat from a wide range of respiratory infections. Additional studies in cats with this group of agents will be necessary to establish this possibility.

The fact that the viruses of group 2, represented by C-27, have been isolated eight times in such widely separated areas as Washington, D.C., Indiana, Oklahoma, and Washington State suggests they may have a wider distribution and more frequent occurrence than the other isolates. As shown by Crandell's experimental work with susceptible cats, as well as by the difficulty we experienced in producing hyperimmune rabbit serum, these viruses failed to elicit a good antibody response. In preliminary experiments with cats, using another strain of serotype C-27, we found that only traces of antibody were produced following intranasal inoculation. Thus, further evaluation of the importance of this virus in the field may have to depend on the frequency with which isolations can be obtained, unless some more sensitive serologic procedure is devised. Evaluation of potential vaccines against this agent may also have to be determined by means other than the neutralization test.

The 3 viruses (KCD, F-20, and FPL) isolated from spleen tissue, while obtained

from cats showing signs of feline enteritis, are not the causative agent of that disease, and their possible role in feline diseases must await further study.

SUMMARY

Twenty-three new feline viruses isolated from cats were studied for antigenic relationship by the cross-neutralization test. Eight were found to be antigenically identical to the C-27 virus of Crandell described previously. The remainder appeared to be different serologic prototypes, although some elicited low serologic cross-relationships. Their possible significance in the feline respiratory disease picture is discussed.

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SUMMARIO IN INTERLINGUA

Relation Serologic de Nove Virus Cytopathogenic Felin

Vinti-tres nove virus felin isolate ab cattos esseva studiate pro lor relation antigenic per le test de neutralisation cruciate. Esseva trovate que octo esseva antigenicamente identic con virus C-27 de Crandell previemente describite. Le alteres pareva esser differente prototypos serologic, ben que plures evocava debile relationes serologic cruciate. Lor signification possibile in le pathologia respiratori felin es discutite.

Experimental Studies on a New Feline Virus

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PRIOR TO 1957, there were no reports concerning the propagation of feline viruses in cell culture. Further, the only confirmed virus diseases of cats were rabies, [19] feline panleukopenia,[10,11,15] and feline pneumonitis.[1,2,12] Although reports of three other feline virus diseases (feline virus pneumonia,[3] feline influenza,[14] and viral encephalopathy [18,22]) are in the literature, it is our opinion that their true identity has yet to be established.

More recently, however, two new feline viruses have been reported, and the virus of panleukopenia has been reported as propagating in tissue cultures of feline kidney origin. Early in 1957, Fastier [7] isolated a new feline virus in New Zealand, designated "kidney cell degenerating virus" (KCD). Later in the same year, Bolin [4] reported on the cultivation of panleukopenia virus in feline kidney tissue culture. In 1958, Crandell and Maurer [5] isolated a feline virus associated with intranuclear inclusion bodies in tissue cultures of feline kidney. Crandell and Despeaux [6] recently proposed the name "feline viral rhinotracheitis" (FVR) for this virus.

This report concerns the isolation of a cytopathogenic agent from a domestic cat that differs from all previously described feline viruses. We have referred to the agent throughout this paper as the California feline isolate (CFI).

MATERIALS AND METHODS

Source of Isolate.—The agent was isolated from the blood and throat of a 9-week-old female cat that developed a fulminating respiratory illness. The early clinical signs consisted of pyrexia (103.8 F.), respiratory rate of 43 to 50 per minute, moist rales on auscultation, anorexia, and lethargy. Evidence of muscular soreness was indicated by a stiff gait and hypersensitivity to touch. Sneezing or coughing was not observed.

Fifty milligrams of tetracycline was administered intramuscularly to the cat on the first and second days of illness. She drank milk on the third and fourth days, ate solid food on the fifth day, and on the sixth day appeared normal. On the seventh day following onset of illness the cat was removed from the laboratory, and no further observations were possible.

Tissue Cultures.—Primary monolayer cultures of kidney tissue from cats, dogs, goats, suckling rabbits, and monkeys were prepared according to the method of Madin *et al.*[16] Cell strains of swine (PK-2a),[23] bovine (MDBK) and ovine (MDOK) kidney cells were prepared as described by Madin and Darby.[17] X-irradiated HeLa cell cultures were prepared according to Puck and Marcus,[20] and FL (human amnion) cells were prepared according to Fogh and Lund.[8] Primary cultures of human amnion were prepared by the method of Zitcer *et al.*[24] Nutrient medium consisted of 0.5 per cent lactalbumin hydrolysate in modified Hanks' balanced salt solution. All mediums contained 5 per cent lamb serum, 500 units of penicillin, 0.5 mg. streptomycin, and 100 units of nystatin* per milliliter.

Isolation Techniques.—On the first day of illness, a blood sample was obtained from the cat and each of three tubes of feline kidney tissue culture was inoculated with 0.3 ml. of blood. Two throat swabs were also made, and one swab was placed in each of two tubes of feline kidney tissue culture. After incubation for four and one-half hours at 37 C., the nutrient fluid containing the blood or throat swab was removed, the monolayer rinsed twice and covered with 1 ml. of medium prior to additional incubation. All subsequent isolations from other cats were done in this manner.

Virus Titrations.—Serial tenfold dilutions of tissue culture fluid containing the agent were prepared with nutrient medium as the diluent; 0.1 ml. of each dilution was added to each of four tubes. After continued incubation at 37 C., all cultures were examined daily for a 72-hour period for the presence or absence of cytopathogenic change (CPC). The end point was calculated by the method of Reed and Muench [21] and expressed as the 50 per cent tissue culture infective dose ($t.c.i.d._{50}$) per 0.1 ml. of tissue culture fluid.

From the Armed Forces Institute of Pathology, Washington, D.C. (Crandell), and Naval Biological Laboratory, School of Public Health, University of California, Berkeley (Madin).

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* Mycostatin, produced by E. R. Squibb and Sons, New York, N.Y.

Serum-Neutralization Tests.—Serial dilutions of serum previously inactivated at 56 C. for 30 minutes were prepared, with nutrient fluid as the diluent. An equal amount of virus suspension diluted to contain approximately 1,000 tissue culture infective doses was added to each dilution. The serum-virus mixtures were then incubated at room temperature for one hour. After incubation, 0.1 ml. of each serum-virus mixture was inoculated into each of three feline kidney culture tubes. The tubes were incubated at 37 C., and daily observations for cytopathogenic changes were made. The presence of neutralizing antibodies was indicated by the absence of cytopathogenic changes. After five days of incubation, the highest serum dilution (final dilution after mixing with virus) that inhibited the CPC in two or more tubes was considered to be the antibody titer.

Immune Serum Production.—Young adult New Zealand rabbits were used for production of immune serum. Each was given three intravenous inoculations of 5 ml. of undiluted infected tissue culture fluid at weekly intervals. Blood samples were obtained prior to inoculation and again one week after the last injection. As a control for nonspecific inhibition of CPC, 1 rabbit was given tissue culture fluid from uninfected cultures.

Immune serums were prepared against Bolin's panleukopenia virus, FVR virus, and the new isolate. A panleukopenia immune serum was produced by inoculating a rabbit with two subcutaneous doses of a commercial vaccine at weekly intervals, followed by one intraperitoneal injection of 1 ml. of live virus-infected tissue. Rooster immune serum against feline pneumonitis was obtained through the courtesy of Dr. Francis Gordon, Naval Medical Research Center, Bethesda, Md. Rabbit immune serum against Fastier's KCD virus was obtained through the courtesy of Dr. John Gorham, Fur Animal Disease Research Laboratory, U.S. Department of Agriculture, Pullman, Washington.

Animal Inoculation.—Ten- to 12-week-old domestic cats, 3- to 4-week-old mice of the Namru [9] and Webster strains, and 7-day embryonating eggs were used. The cats were allotted to three groups, each group consisting of 3 animals. In groups 1 and 2, 1 of the 3 cats was used as a control. Each cat in group 1 was given 0.25 to 0.5 ml. of third tissue culture passage virus intranasally. Undiluted virus of the twelfth tissue culture passage was used in group 2; 1 cat was inoculated intranasally with 0.25 ml. and 1 was given 1.0 ml. intramuscularly. The cat used as a contact control for this group was placed in the same cage with the other 2. The 3 cats in group 3 were each given 0.25 to 0.5 ml. of undiluted 30th tissue culture passage virus by the intranasal route. Cats were inoculated under pentothal anesthesia.

For the Namru mice, the inoculations with 42nd-passage virus were: 0.05 ml. intranasally, 0.5 ml. intraperitoneally, and 0.03 ml. intracerebrally. The Webster mice were given 0.05 ml. of 32nd-passage virus suspension instilled intranasally with four consecutive blind passages of 20 per cent lung suspension.

During the observation period prior to inoculations, rectal temperatures and (with the exception of the animals in group 2) white blood cell counts were taken daily. All temperatures and white blood cell counts were considerd to be within the normal limits. Nasal smears were taken and assayed for the presence of cytopathogenic agents in tissue culture. No cytopathogenic agents were isolated from nasal smears. All preinoculation serums were negative for antibodies that were specific against the agent, as evidenced by their failure to inhibit cytopathogenic changes in tissue culture.

Preparations for Histologic Examination.—Feline renal cells grown on cover slips were fixed with Bouin's fluid and stained with hematoxylin and eosin or Giemsa's stain.

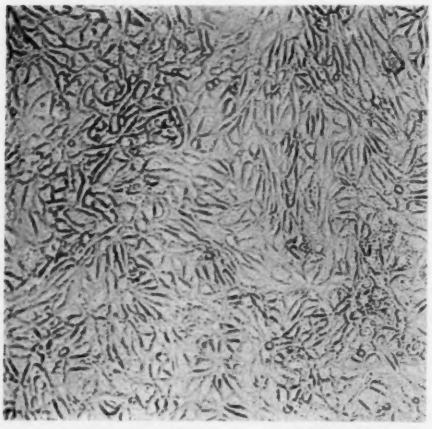

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Fig. 1—Uninoculated tissue culture of feline kidney. Unstained; x 100.

RESULTS

Cytopathogenic Change in Feline Kidney Tissue Cultures.—The feline kidney cultures inoculated with the blood and throat swabs showed cytopathogenic changes 68 hours after inoculation.

The cytopathogenic changes as observed under low-power (x 100) in the unfixed preparation appeared as small scattered foci of degenerative cells which progressed until eventually the entire monolayer was involved, causing the cells to be released from the glass. The degeneration consisted of a rounding and shrinking of the cells with a tendency to form clusters (fig. 1, 2).

In stained preparations, various stages of cell degeneration were observed. Most

cells were rounded. However, some were spindle-shaped, with beaded protoplasmic processes (fig. 3). Affected cells contained either pyknotic or karyolytic nuclei. When stained with hematoxylin and eosin, the nuclear chromatin was intensely basophilic and the cytoplasm was dark red and somewhat granular. No inclusion bodies or elementary bodies were demonstrated. To date, the CFI agent has been serially passaged 56 times in tissue cultures of feline kidney cells without loss of cytopathogenicity. Readily visible plaques were produced when the virus was inoculated on monolayers of feline kidney cultures following the method of Hsiung and Melnick.[13]

Transmission Studies in Cats.—In group 1, all the cats had a febrile response of 103 to 104 F. between the fourth and fifth days. With 1 exception, there were no other clinical signs. On the fourth postinoculation day, 1 cat had a febrile response of 104 F. and held one front paw as though in pain. On the fifth day, a slight nasal and ocular discharge was present. One day later, however, his temperature was considered normal, and other signs of illness were absent. The white blood cell counts remained within normal limits. A cytopathogenic agent was recovered in tissue culture from throat swabs taken on the fifth day following inoculation. The cats were observed for 30 days, at which time convalescent serum was obtained. The control, which was held in a separate room, remained normal throughout this period of observation.

In group 2, the temperatures of the cats ranged from 101.6 to 104.4 F. between the first and fourth days following exposure. Both the inoculated cats and the contact cat showed clinical signs similar to those observed in the animal from which CFI was originally isolated. The cat that was inoculated via the intranasal route also showed ulcerative lesions of the mouth and nares. There was a mucous discharge followed by a purulent discharge from the mouth, nose, and eyes, and some blood was visible in the feces. The temperature peak of the contact cat was recorded on the third postinoculation day, which was one day later than that observed in the other 2 cats.

Ten days after the inoculation, throat swabs of all 3 cats in group 2 were taken and inoculated into feline renal cell cultures. Cytopathogenic changes were observed within 72 hours after incubation.

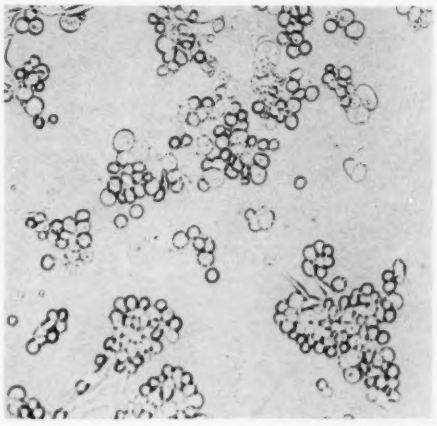

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Fig. 2—Cytopathogenic changes in culture infected with feline virus. Unstained; x 100.

The 3 cats made uncomplicated recoveries from the illness, and all appeared normal within 12 days following inoculation. Throat swabs of all cats, made at two-week intervals for a total of eight weeks after recovery, were negative for cytopathogenic agents, as indicated by its absence of cytopathogenic effects in feline renal cell cultures. Blood samples were obtained from all 3 animals for preparation of postinoculation serums 46 days after the initial inoculation.

In group 3, the cats did not develop clinical signs. However, on the first day following inoculation there was a slight rise in temperature, averaging from 0.9 to 1.5 degrees higher than before inoculation. The white blood cell counts remained normal.

Neutralization Tests.—Neutralization tests conducted on all preinoculation cat serums were negative for neutralizing antibodies specific for CFI in the dilution tested (final 1:5).

The neutralizing end points on postinoculation serums for the various routes of inoculation in the three groups of cats are as follows: group 1 (intranasal), 1:100, 1:1,000, and >1:10; group 2, greater than 1:500 in each animal; and group 3 (intranasal), 1:20, 1:80, and 1:20.

Rabbit serums prepared against the different viruses had the following neutralizing titers against the homologous virus:

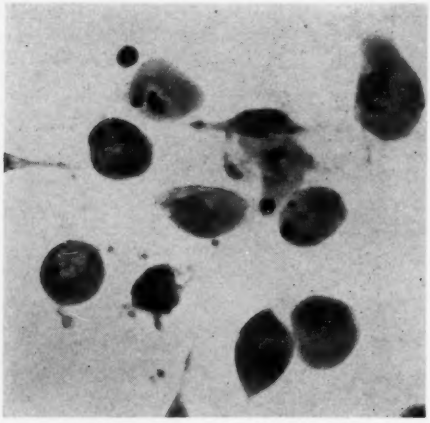

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Fig. 3—Cytopathology of feline kidney cells infected with feline virus. H & E stain; x 630.

CFI, 1:40; FVR virus, 1:64; and Bolin's virus, 1:40. The immune rabbit serum prepared against the uninfected tissue culture fluid did not inhibit the cytopathogenic effect of any of the viruses.

Cross-neutralization tests conducted with these three viruses and their homologous immune serums showed no cross protection in the lowest serum dilution tested (final dilution 1:5). Rooster antiserum against feline pneumonitis did not neutralize CFI in final dilutions of 1:2 or greater in tissue cultures of feline kidney. The rabbit serum prepared against the commercial panleukopenia vaccine failed to inhibit the cytopathogenic effect of the CFI virus. The KCD virus antiserum also failed to neutralize the CFI virus; however, only serum containing 10 neutralizing antibody units was available.

Although this agent was isolated from a cat in the San Francisco Bay area in California, kittens in the eastern part of the United States possessed antibodies against it. In one antibody survey, serums from 16 apparently normal kittens, 10 to 14 weeks of age, were tested. For screening purposes, serum dilutions of 1:2 and 1:4 were tested against approximately 1,000 t.c.i.d.$_{50}$ doses of the virus. Of these 16 serums, three completely inhibited the CPC in both dilutions tested, two serums showed partial inhibition in one or the other dilution, and 11 showed no inhibition in the lowest dilution tested.

Host Range Studies.—Namru mice were inoculated via the intranasal, intracerebral, and intraperitoneal routes with 42nd-passage material. No signs were observed in the mice during the 33-day observation period. A group of Webster strain mice were inoculated intranasally with the 32nd-passage material. Four blind passages were made at 4- to 5-day intervals, each with 0.05 ml. of a 20 per cent lung suspension instilled intranasally. Lung impression smears were prepared and stained by the Macchiavello method. The lungs were free of macroscopic lesions, and all smears were negative for elementary bodies. In addition, three attempts to infect chicken embryos via the allantoic and yolk sac routes showed equivocal results; *i.e.*, nonuniform susceptibility and unsuccessful passages. Three blind passages of the agent in tissue cultures of dog, goat, suckling rabbit, and monkey kidney, and in FL, HeLa, HeLa x-irradiated, and human amnion cells produced no cytopathogenic effects.

Preliminary experiments with hemagglutination tests using red blood cell suspension from a cat, sheep, rat, day-old chick, rabbit, group O human, pig, mouse, frog, hamster, guinea pig, snake, and ferret were all negative.

The agent has been successfully lyophilized. Prior to lyophilization, the t.c.i.d.$_{50}$ was $10^{-6.0}$; immediately after lyophilization, two reconstituted samples showed t.c.i.d.$_{50}$ values of $10^{-5.8}$ and $10^{-5.9}$. The lyophilized samples had a t.c.i.d.$_{50}$ of $10^{-5.5}$ after storage for five months at −25 C., indicating the stability of this agent under these conditions. The virus in tissue culture fluid stored at −20 C. and −60 C. for 18 months did not show an appreciable loss of titer.

DISCUSSION

Of major importance in studying a new isolate is its relationship to the presently recognized viruses for the given species. After this relationship has been established, the significance of the isolate as a causative agent of disease for that species should be determined. In regard to the first consideration, evidence is presented to show that this agent is different from the previously reported feline viruses. As for the second, the results of our study support the possibility that this agent may be the

cause of a nonfatal respiratory infection in cats. However, it is emphasized that additional transmission studies with this new agent are needed to more fully understand its relationship to the feline respiratory virus disease complex.

The recognized feline viruses to which this agent was compared are feline pneumonitis (Baker), panleukopenia, the kidney cell degenerating virus (KCD), and the FVR virus.

The virus of feline pneumonitis, when propagated in mice and embryonating eggs, forms elementary bodies [2] characteristic of the psittacosis-lymphogranuloma group. All attempts to infect these with the CFI agent have been unsuccessful. Furthermore, elementary bodies have not been demonstrated in tissue culture preparations stained with either Giemsa's or Macchiavello's stains. In addition to these differences, immune serum specific against feline pneumonitis did not inhibit the cytopathogenic effect of this agent in tissue cultures of feline kidney.

Panleukopenia was not observed in the two groups of cats (1 and 3) in which white blood cell counts were taken. In the other group (2), the resulting infection was nonfatal as compared to the high mortality rate observed with panleukopenia.

There was no antigenic relationship between this agent and Bolin's panleukopenia virus as evidenced by *in vitro* cross-neutralization tests. Rabbit serum prepared against panleukopenia virus did not inhibit the cytopathogenic effect of the CFI isolate in cultures of feline kidney cells.

The cytopathogenicity of the CFI virus in tissue culture differs from that of the FVR virus. Intranuclear inclusion bodies, which are a characteristic feature of the FVR virus, are not found with the CFI agent. *In vitro* cross-neutralization tests revealed no antigenic similarities between these two agents.

Rabbit immune serum against the KCD virus did not neutralize the CFI virus. The cytopathogenicity of the KCD virus for feline kidney tissue cultures, as described by Fastier,[7] closely resembles that seen with the CFI isolate. The CFI virus is much more stable upon storage than the KCD virus reported by Fastier.

The results of the transmission studies support the hypothesis that this agent, under the proper conditions, is capable of producing clinical disease in susceptible cats. The 3rd tissue culture passage of this agent produced a mild, febrile, clinical response in a group of inoculated cats, with the development of neutralizing antibodies. Cats inoculated with the 12th tissue culture passage developed a more severe illness with a higher antibody titer than those in the first group. A third group of cats given the thirtieth tissue culture passage of virus showed no clinical response, but an antibody titer against the agent was produced.

These differences in response in the three groups of cats suggest that the agent had undergone some alteration upon serial passage in feline kidney tissue cultures. Between the 3rd and 12th passages its virulence for the cat was enhanced. Following subsequent passage in feline tissue cultures, the agent was attenuated so that it was no longer pathogenic for the cat. During the time these passages were made in tissue culture, there was no change in the cytopathogenicity of the virus for feline tissue cultures, nor were there any significant differences in the $t.ci.d._{50}$ of any one passage tested. Although these experiments are not conclusive evidence that the pathogenicity of this agent was altered, the results do follow the pattern of attenuation demonstrated by some viruses upon serial transfer in tissue culture. Additional studies are needed to determine its full significance as a pathogen for the domestic cat.

SUMMARY

California feline isolate (CFI), a new cytopathogenic agent of feline origin capable of producing a nonfatal respiratory infection in domestic cats, is described. A comparison of this agent with the viruses responsible for feline pneumonitis (Baker), kidney cell degenerating virus (KCD), feline panleukopenia, and the feline viral rhinotracheitis (FVR) virus shows that it is not related either antigenically or by the other criteria used in this study.

This agent has been carried through a total of 56 serial passages in cell cultures of feline kidney without loss of cytopathogenicity. Loss of virulence for experimental cats was found to occur between the 12th and the thirtieth passage without loss of antigenicity. The California feline isolate may be readily lyophilized and can be stored at −20 C. and −60 C. for at least 18 months with little loss of infectivity titer.

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SUMMARIO IN INTERLINGUA

Studios Experimental in Un Nove Virus Felin

Es describite, sub le designation "isolato felin California," un nove agente cytopathogenic de origine felin que es capace de producer un non-letal infection respiratori in le catto domestic. Un comparation del agente con le virus responsabile pro pneumonitis felin (Baker), degeneration reno-cellular, panleucopenia felin, e felin rhinotracheitis virusal demonstra que illo es distincte tanto antigenicamente como etiam secundo le altere criterios usate in le presente studio.

Le agente esseva portate a transverso 56 passages serial in cytoculturas renal felin sin perdita de pathogenicitate. Perdita de virulentia pro cattos experimental esseva constatate inter le dece-secunde e le trentesime passage. Isto non esseva accompaniate de perdita de antigenicitate. Le isolato felin California es prestemente lyophilisabile. Illo pote esser immagasinate a −20 e −60 C durante al minus 18 menses sin abassamento del titro de infectivitate.

Effects of Meperidine, Promazine, and Promethazine Preanesthesia on Traumatic Shock and Experimental Pentobarbital Anesthesia in Cats

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THE SYNDROME known as shock has been described, but incompletely understood, for hundreds of years. Following the discovery of its different causes and measurement of physiologic alterations, many therapeutic recommendations were made. The observation that epinephrine and norepinephrine aggravated certain types of bacterial and traumatic shock laid the foundation for investigation of the effects of adrenolytic drugs in shock. Most experimental studies with adrenolytic drugs have been with drum shock in rats and hemorrhagic shock in dogs.

The purpose of this investigation was to determine if the adrenolytic drugs (promazine* and promethazine*) alone or with meperidine**) administered prior to induction of anesthesia with sodium pentobarbital would significantly alter blood pressure, oxygen consumption, hematocrit values, blood volume, circulation time, body temperature, pulse, and respiratory rate of cats subjected to 30 minutes of bowel manipulation.

MATERIALS AND METHODS

Large, adult, uneastrated cats, free from infections and contagious diseases, were used. The methods employed for the administration of the preanesthetic and anesthetic agents were the same as previously described.[5] Ten cats were used in each group. The blood pressure was recorded directly from the aorta (posterior to the renal artery) by means of a mercury manometer and polyethylene tubing. Approximately 5 mg. of heparin was added to the physiologic saline solution used in the manometer. Blood pressure was recorded prior to and immediately after trauma and then every half hour thereafter for four hours. Only the recordings taken before trauma, immediately after trauma, at the lowest point after trauma, and at the end of the four-hour period are shown (tables 1A–1C).

Oxygen consumption was determined with a Phipps and Bird spirometer (1.5-liter capacity) and kymograph utilizing a soda-lime absorber in the system. The trachea, exposed by a midline incision through the skin, subcutis, and cervical muscles, was entered by means of a longitudinal incision and a plastic tube was ligated in place. Cats were allowed to breathe pure oxygen for approximately four minutes to obtain a uniform slope in the kymograph tracing. Recordings of oxygen consumption were made before, immediately following, and four hours after trauma.

Hematocrit values were determined by centrifuging heparinized capillary tubes for five minutes at 11,000 r.p.m. in an International hematocrit centrifuge.[9] Blood samples were taken before and after trauma, and at the end of four hours for hematocrit determinations.

The plasma volume was determined by injecting radioactive iodine (I^{131}) into the venous circulation, as outlined by several authors.[8, 12, 20] The blood volume was calculated from the plasma volume according to the following formulas[35]:

$$\text{Plasma Volume (ml.)} = \frac{\text{Radioactivity of the sample injected (0.5 ml.)}}{\text{Radioactivity of the plasma sample (0.5 ml.)}}$$

$$\text{Hematocrit} = 100 \times 1 - \frac{\text{Blood volume (ml.)}}{\text{Plasma volume (ml.)}}$$

The blood samples were counted at the Department of Biophysics, College of Medicine, University of Minnesota, in a sodium iodide (Thallium) scintillation-well counter. The background counts (made before and after trauma) were averaged and subtracted from each counting.

The blood circulation time was measured by first injecting I^{131} into the posterior vena cava and then measuring the time required for complete circulation with a logarithmic Esterline-Angus recorder. The aperture of the scintillation ratemeter, which was completely transistorized and contained a sodium iodide crystal, was placed over the tho-

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* Sparine (promazine) and Phenergan (promethazine) are produced by Wyeth Laboratories, Inc., Philadelphia, Pa.

** Demerol (meperidine) is produced by Winthrop-Sterns, Inc., New York, N.Y.

racic inlet. In most instances, the time required for the first few circulations could be calculated directly from the recording paper by measuring the distance between the periodic increments in radioactive energy. In other recordings, the troughs before or after the peaks were more clearly defined and used in the calculation of the circulation time. The circulation time was calculated before trauma and at the end of four hours. It was hoped that recordings immediately after trauma could also be made, but the radioactive background, exposure of personnel, and limitations of the equipment made this impracticable.

The colonic and esophageal temperatures were taken every half hour during the 4-hour experiments. The average of these two readings, before trauma and at the end of four hours, is included.

The pulse rate and respirations per minute were recorded every half hour. These data are listed for the values before and after trauma, along with the lowest value for the four-hour period and at the end of four hours.

Intestinal trauma was produced by 30 minutes of intestinal manipulation. This type and amount of trauma has been found adequate to produce irreversible shock in the dog.[1, 4, 25]

RESULTS

The effects of subcutaneous administration of the preanesthetic drugs, meperidine, promazine, and promethazine, prior to pentobarbital anesthesia on traumatic shock are shown (tables 1A–1C). Differences between pre- and post-trauma values of blood pressure, oxygen consumption, and other measurements are listed as plus (+) and minus (−). In all groups, the mean of the values for the 10 cats used in each experiment is included with the standard error of the mean. The latter was calculated according to Trelease.[32] In cats given preanesthetic drugs, the level of significance was compared to cats given only pentobarbital. The level of significance between the means was compared by the method outlined by Snedecor.[29] The critical level of significance of less than one per cent was used in these studies.

Pentobarbital Anesthesia.—The amount of pentobarbital (40 mg./kg.) used per kilogram of body weight was greater for these cats than that used in a previous study (33 mg./kg.)[6] because it was found in early trials that a deeper level of anesthesia (stage III, plane IV, rather than stage III, plane III) was required to keep the animals immobilized during the four-hour period of observation. For this reason, comparisons of the anesthetic dose of pentobarbital can be made within these experiments but not with other studies. Only male cats were used in this experiment, while nonpregnant female cats were used in the earlier investigation.

The mean blood pressure of animals

TABLE 1A—A Comparison of the Effect of Various Preanesthetics on Pentobarbital Anesthesia and Shock

	Wt. (kg.)	Pentobarbital (mg./kg.)	Blood pressure (mm. Hg) Pre-shock	Post-shock	4 hr. low	"End" 4 hr.	O_2 (ml./kg./min.) Pre-shock	Post-shock	"End" 4 hr.
GROUP 1—PENTOBARBITAL									
Mean	2.8	40.0	123.0	−25.0	−42.0	−31.0	4.8	−1.0	+0.7
± s.e.		2.1	5.3	5.4	6.8	7.1	0.36	0.21	0.45
GROUP 2—MEPERIDINE (11 MG./KG.) PENTOBARBITAL									
Mean	3.5	36.0	121.0	−12.0	−30.0	+21.0	5.7	−1.7	−1.2
± s.e.		1.6	9.1	8.5	13.6	10.1	0.29	0.29	0.25
Prob.(P)		0.10–0.20	>0.50	0.20–0.30	0.40–0.50	0.20–0.30	0.10–0.20	>0.50	>0.01
GROUP 3—PROMAZINE (4.4 MG./KG.) PENTOBARBITAL									
Mean	3.4	38.0	113.0	−23.0	−31.0	−11.0	5.4	−1.4	−1.5
± s.e.		2.3	9.9	7.2	8.5	12.5	0.22	0.38	0.31
Prob.(P)		>0.50	0.30–0.40	>0.50	0.30–0.40	0.10–0.20	0.10–0.20	0.30–0.40	<0.01
GROUP 4—MEPERIDINE (11 MG./KG.) PROMAZINE (4.4 MG./KG.) PENTOBARBITAL									
Mean	3.4	28.0	101.0	−13.0	−21.0	−1.0	5.0	−1.1	−0.8
± s.e.		2.7	6.7	5.8	8.1	12.0	0.33	0.54	0.70
Prob.(P)		<0.01	0.02–0.05	0.10–0.20	0.05–0.10	0.02–0.05	>0.50	>0.50	0.05–0.10
GROUP 5—PROMPTHAZINE (8.8 MG./KG.) PENTOBARBITAL									
Mean	3.4	35.0	119.0	−14.0	−23.0	+14.0	5.4	−0.6	−0.2
± s.e.		2.6	9.5	3.6	4.3	7.1	0.20	0.32	0.48
Prob.(P)		>0.50	>0.50	0.10–0.20	0.20–0.05	<0.01	0.10–0.20	0.30–0.40	0.10–0.20
GROUP 6—MEPERIDINE (11 MG./KG.) PROMETHAZINE (8.8 MG./KG.) PENTOBARBITAL									
Mean	3.3	28.0	122.0	−31.0	−45.0	−5.0	4.9	−1.4	−0.6
± s.e.		1.4	13.3	7.3	8.5	10.4	0.53	0.17	0.38
Prob.(P)		<0.01	>0.50	>0.50	>0.50	0.05–0.10	>0.50	0.40–0.50	0.02–0.05

under pentobarbital anesthesia was 123 mm. of mercury. This compares closely with the carotid blood pressure of 120 mm. Hg which was reported by Huidobro.[14] The blood pressures after trauma and at the end of four hours were similar. The average of the lowest blood pressures during the four-hour period was lower than immediately after trauma or at the end of the four-hour period. This appears to be an aggravation of hypotension during the post-trauma period and a partial recovery at the end of the four hours.

The oxygen consumption before intestinal manipulation was 4.8 ml. per kilogram per minute and agrees with data reported by Baxter *et al.*[3] After intestinal manipulation, the oxygen consumption decreased. Despite this, it increased above the pretrauma level at the end of the four-hour period.

The packed cell volume (PCV) of cats under pentobarbital anesthesia was 39 per cent. Jarcho,[15] who reported a preanesthetic PCV of 40 per cent, found that the hematocrit volume decreased 27 per cent after cats were anesthetized. Sequestration of erythrocytes by the spleen was considered to be the cause of this reduction. Trauma to the spleen or the injection of epinephrine has been observed to decrease splenic size following pentobarbital anesthesia. Since the pretrauma blood samples were taken after the abdomen was opened and the aorta was cannulated, it is possible that this barbiturate effect on the spleen and the PCV was diminished. However, comparison between groups should still be valid because blood samples were taken from each group of animals in the same order.

The blood volume of cats anesthetized with pentobarbital has been reported as 43 ml./kg.[11] In this study, the blood volume before trauma was 58 ml./kg., and 50 ml./kg. four hours after shock.

Under pentobarbital anesthesia, the circulating times of 2 dogs have been reported as 9.7 and 9.8 seconds.[10] These values increased to 23.3 and 24.5 after traumatic shock. The mean complete circulating time in the anesthetized cats was 7.4 seconds. This increased 1.3 seconds following trauma.

The body temperature before and after trauma is included to illustrate the fall in temperature over the four-hour period. This decrease in temperature depends on the duration of anesthesia, depth of anesthesia, and the temperature of the environment, rather than any specific drug action. Obviously, the fall in temperature would be greater in an animal in which the peri-

TABLE 1B—A Comparison of the Effect of Various Preanesthetics on Pentobarbital Anesthesia and Shock

	Packed cell volume			Blood vol. (ml./kg.)		Circ. time (sec.)		Temp. (C.)	
	Pre-shock	Post-shock	"End" 4 hr.	Pre-shock	"End" 4 hr.	Pre-shock	"End" 4 hr.	Pre-shock	"End" 4 hr.
GROUP 1—PENTOBARBITAL									
Mean	39.0	+7.0	+11.0	58.0	−8.0	7.4	+1.3	36.0	−5.0
± s.e.	1.3	1.5	1.9	1.8	1.6	0.29	0.53		
GROUP 2—MEPERIDINE (11 MG./KG.) PENTOBARBITAL									
Mean	40.0	+5.0	+7.0	59.0	−9.0	8.1	+0.8	37.0	−5.0
± s.e.	4.4	0.9	1.1	2.3	1.6	0.55	0.43		
Prob. (P)	>0.50	0.20–0.30	0.05–0.10	>0.50	>0.50	0.30–0.30	0.40–0.50		
GROUP 3—PROMAZINE (4.4 MG./KG.) PENTOBARBITAL									
Mean	40.0	+5.0	+9.0	57.0	−6.0	8.1	+1.8	36.0	−5.0
± s.e.	2.8	2.4	2.5	2.5	2.6	0.63	0.47		
Prob. (P)	>0.50	0.40–0.50	>0.50	>0.50	>0.50	>0.50	0.40–0.50		
GROUP 4—MEPERIDINE (11 MG./KG.) PROMAZINE (4.4 MG./KG.) PENTOBARBITAL									
Mean	40.0	+8.0	+12.0	60.0	−9.0	7.5	+0.4	36.0	−5.0
± s.e.	1.7	1.9	2.2	1.6	1.7	0.44	0.37		
Prob. (P)	>0.50	>0.50	>0.50	0.40–0.50	>0.50	>0.50	0.10–0.20		
GROUP 5—PROMETHAZINE (8.8 MG./KG.) PENTOBARBITAL									
Mean	40.0	+14.0	+11.0	63.0	−10.0	7.8	+1.3	35.0	−2.0
± s.e.	2.1	1.1	1.9	1.7	0.7	0.45	0.74		
Prob. (P)	>0.50	0.10–0.20	>0.50	0.05–0.10	0.20–0.30	0.40–0.50	>0.50		
GROUP 6—MEPERIDINE (11 MG./KG.) PROMETHAZINE (8.8 MG./KG.) PENTOBARBITAL									
Mean	38.0	+6.0	+8.0	60.0	−10.0	8.5	−1.3	35.0	−4.0
± s.e.	2.6	1.3	1.8	2.4	2.4	0.49	0.50		
Prob. (P)	>0.50	>0.50	0.40–0.50	>0.50	0.40–0.50	0.05–0.10	>0.50		

toneal cavity was exposed than in one in which there had been no operative procedure.

The heart rate in cats is subject to wide variation. In this series, there were 153 beats per minute in cats after anesthetization with pentobarbital. After trauma, the heart rate decreased by 20 beats per minute. The lowest mean number of beats was 27 less than the pretrauma average, but there were only 15 less at the end of four hours.

Wang and Nims [33] found that respiratory rate was 19 per minute after administration of pentobarbital. Prior to clinical surgery, the respiratory rate varied from 10 to 20. In this series, there were 12 respirations per minute before and 11 after intestinal manipulation. The lowest number of respirations during the four-hour period was nine, and there were 16 respirations per minute after four hours.

Meperidine-Pentobarbital Anesthesia.—The only significant difference between cats in this group and those anesthetized with pentobarbital alone was a decrease in the oxygen consumption four hours after trauma and an elevation in heart rate prior to trauma. The oxygen consumption was 0.9 ml./kg./min. more in the cats given meperidine than in the control cats prior to trauma and 1.0 ml./kg./min. less in these respective groups four hours after trauma. This trend will be discussed later. The heart rate was significantly greater prior to trauma in those animals which were premedicated with meperidine. The fact that pentobarbital has a cardiotoxic effect and that the administration of meperidine spares higher doses of this drug may partially explain this result.

Promazine-Pentobarbital Anesthesia.—The only significant features of this preanesthetic drug were the decrease in oxygen consumption four hours after trauma and an increase in the number of respirations prior to trauma. Meperidine and promazine alone significantly reduced the oxygen consumption four hours after trauma; they did not produce a significant reduction in combination. This reduces the value of these initial observations.

Meperidine-Promazine-Pentobarbital Anesthesia.—The only significant finding in these cats was the reduction in the anesthetic dose of pentobarbital which has been previously reported.[5]

Promethazine-Pentobarbital Anesthesia.—The blood pressure four hours after trauma was significantly elevated and the pre-

TABLE 1C—A Comparison of the Effects of Various Preanesthetics on Pentobarbital Anesthesia and Shock

	Pulse (min.)				Respirations (min.)			
	Pre-shock	Post-shock	4 hr. low	"End" 4 hr.	Pre-shock	Post-shock	4 hr. low	"End" 4 hr.
GROUP 1—PENTOBARBITAL								
Mean	153.0	−20.0	−27.0	−15.0	12.0	−1.0	−3.0	+4.0
± s.e.	7.0	6.8	6.4	7.2	1.3	0.9	1.3	2.9
GROUP 2—MEPERIDINE (11 MG./KG.) PENTOBARBITAL								
Mean	174.0	−12.0	−30.0	−3.0	11.0	+1.0	−2.0	+4.0
± s.e.	4.4	5.3	8.2	6.2	1.6	1.5	2.1	2.2
Prob. (P)	<0.01	0.30–0.40	>0.50	0.20–0.30	>0.50	0.20–0.30	>0.50	>0.50
GROUP 3—PROMAZINE (4.4 MG./KG.) PENTOBARBITAL								
Mean	160.0	−12.0	−20.0	−7.0	21.0	−3.0	−4.0	+3.0
± s.e.	4.6	5.1	4.6	6.7	2.1	1.6	1.5	2.0
Prob. (P)	0.30–0.40	0.40–0.50	0.30–0.40	0.40–0.50	<0.01	0.20–0.30	>0.50	>0.50
GROUP 4—MEPERIDINE (11 MG./KG.) PROMAZINE (4.4 MG./KG.) PENTOBARBITAL								
Mean	161.0	−6.0	−23.0	−13.0	10.0	+1.0	0.0	+8.0
± s.e.	3.6	5.6	6.9	8.8	1.3	0.94	1.3	2.1
Prob. (P)	0.30–0.40	0.10–0.20	>0.50	>0.50	0.20–0.30	0.10–0.20	0.10–0.20	0.20–0.30
GROUP 5—PROMETHAZINE (8.8 MG./KG.) PENTOBARBITAL								
Mean	168.0	−10.0	−22.0	−2.0	19.0	+1.0	+1.0	+9.0
± s.e.	4.5	6.2	7.1	9.4	1.7	0.57	1.2	1.5
Prob. (P)	0.05–0.10	0.20–0.30	>0.50	0.20–0.30	<0.01	0.05–0.10	0.02–0.05	0.05–0.10
GROUP 6—MEPERIDINE (11 MG./KG.) PROMETHAZINE (8.8 MG./KG.) PENTOBARBITAL								
Mean	166.0	−21.0	−32.0	+4.0	17.0	+1.0	−1.0	+6.0
± s.e.	12.8	10.2	8.1	8.6	1.2	2.2	1.9	2.0
Prob. (P)	0.30–0.40	>0.50	>0.50	0.10–0.20	0.01–0.02	0.30–0.40	0.40–0.50	>0.50

trauma respirations were significantly increased.

Meperidine-Promethazine-Pentobarbital Anesthesia.—The only significant observation was that the anesthetic dose of pentobarbital was reduced after administration of these drugs. The elevation of the number of respirations was almost significant at the one per cent level; blood pressure was not significantly elevated.

Discussion

The effect of preanesthetic and anesthetic drugs on shock in the cat has been given little attention. In other experimental animals, the difficulty of separating the depressant effects of drugs from those of shock-inducing procedure have limited such investigations. Early in the study of shock, pain was observed to be an aggravating factor, and ether, which has a strong, analgesic action, was widely used to prevent shock. Following the introduction of the barbiturates, it was demonstrated in dogs that the onset of traumatic shock, due to intestinal manipulation, and subsequent death were delayed longer when sodium amobarbital was used as the anesthetic than when ether was employed.[28] Kendrick and Uihlein [19] and Pender and Essex [25] found pentobarbital safer than ether in avoiding experimental shock. Moon [21,22] compared barbiturates and ether and stated that dogs under ether anesthesia could withstand more trauma without developing shock than those anesthetized with derivatives of barbituric acid. Other authors [24,26] found no significant difference in the production of shock in dogs anesthetized with barbiturates or with ether. The hypotension produced in the cats in this experiment is milder than has been reported in dogs subjected to the same amount of bowel manipulation.[1,4,25]

Aub [2] found a 19 per cent reduction in caloric utilization in cats after mild shock, with an increase to normal or above normal during the recovery stage. Increased oxygen consumption in this period has been attributed by Cuthbertson [7] to the onset of "traumatic inflammation." Due to either a lighter plane of anesthesia or traumatic inflammation, the oxygen requirements of cats in nearly all the groups increased at the end of the four-hour period. It might be concluded that the lighter plane of anesthesia is more important in those experiments because the increase in oxygen consumption paralleled the increase in the number of respirations, which is a reliable sign of awakening from anesthesia.

Radioactive iodine was used in both the determination of the blood volume and the circulation time. In order to minimize the amount of radioactive isotope used and to conserve blood during the period of observation, it was decided to measure the blood volume and circulation time only at the end of the four-hour period. The decrease in blood volume would be greater at this time than immediately after shock because more time will have been allowed for the plasma and erythrocytes to escape into the traumatized tissue.

Hershey *et al.* [12] demonstrated that chlorpromazine, a phenothiazine derivative similar to promazine and promethazine, significantly increased the survival time of rats subjected to graded hemorrhage. Other authors [4,15,17,18,20,23,27,34,36] have reported that chlorpromazine has a beneficial effect in hemorrhagic and traumatic shock, although Millican and Rhodes [20] did not observe that promethazine significantly protected mice against tourniquet trauma. The studies of Thomas [31] and Zweifach *et al.*[37] have indicated that a lethal synergism exists between gram-negative endotoxins and epinephrine. If the adrenolytic phenothiazine derivatives effectively block the production of epinephrine, this would explain the beneficial role of these drugs in shock.

In cats given preanesthetic medication, there was a significant increase in the number of respirations prior to shock in cats given promazine-pentobarbital. This was probably due to random sampling, since there was not a great difference in the number of preshock respirations when promazine and meperidine were used prior to pentobarbital. In the groups in which meperidine and promazine or meperidine and promethazine were used prior to anesthesia, the anesthetic dose of pentobarbital was significantly reduced. Similar results, using these and other ataractic agents combined with meperidine, have been observed in cats.[6] When promethazine was administered prior to pentobarbital, there was a significant increase in the number of respirations prior to shock. This value for the meperidine and promethazine was also nearly significant. This substantiates the

observation that promethazine does not depress respiration at these dosages. It does not demonstrate this property of the drug in animals in shock since there were no significant changes in the respiratory rate following trauma.

SUMMARY

The preanesthetic administration of meperidine (11 mg./kg.), promazine (4.4 mg./kg.), promethazine (8.8 mg./kg.), meperidine (11 mg./kg.), and promazine (4.4 mg./kg.), or meperidine (11 mg./kg.), and promethazine (8.8 mg./kg.) prior to pentobarbital anesthesia did not consistently improve the blood pressure, oxygen consumption, packed cell volume, blood volume, circulation time, heart rate, or respiratory rate of cats subjected to 30 minutes of intestinal manipulation.

Preanesthetic administration of either meperidine and promazine or meperidine and promethazine did significantly reduce the subsequent anesthetic dose of pentobarbital. When meperidine was administered prior to pentobarbital, the oxygen consumption was remarkably reduced four hours after intestinal manipulation; the heart rate was elevated before the trauma was produced. With promazine-pentobarbital anesthesia, the oxygen consumption was also significantly reduced four hours after intestinal manipulation; the respiratory rate after anesthetization increased. Neither the oxygen consumption, heart rate, or respiratory rate were significantly changed when meperidine and promazine were administered together.

Promethazine produced a notable increase in the blood pressure four hours after trauma and the respiratory rate was also increased prior to the traumatic procedure. Neither of these functions were significant at the one per cent level when meperidine and promethazine were administered concomitantly, although a similar trend was apparent in both instances.

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SUMMARIO IN INTERLINGUA

Le Effectos de Preanesthesia per Meperidina, Promazina, e Promethazina Super Choc Traumatic e Anesthesia Experimental per Pentobarbital in Cattos

Le administration preanesthetic de (1) meperidina in un dosage de 11 mg per kg de peso corporee, (2) promazina in un dosage de 4,4 mg per kg, (3) promethazina in un dosage de 8,8 mg per kg, (4) meperidina e promazina in dosages de 11 e 4,4 mg per kg, respectivemente, o (5) meperidina e promethazina in dosages de 11 e 8,8 mg per kg, effectuate ante le application de anesthesia per pentobarbital, non meliorava uniformemente le tension de sanguine, le consumo de oxygeno, le volumine de cellulas paccate, le volumine de sanguine, le tempore del circulation, le frequentia cardiac, o le frequentia respiratori in cattos subjicite a 30 minutas de manipulation intestinal.

Le administration preanesthetic de (1) meperidina, (2) promazina, o (3) meperidina e promethazina reduceva significativemente le subsequente dose anesthetic de pentobarbital. Quando meperidina esseva administrate ante le application de pentobarbital, le consumo de oxygeno esseva remarcabilemente reducite quatro horas post le manipulation intestinal. Le frequentia cardiac esseva elevate ante le production del trauma. Quando promazina esseva administrate ante le application de pentobarbital, le consumo de oxygeno esseva etiam reducite significativemente quatro horas post le manipulation intestinal. Le frequentia respiratori post le anesthesia esseva augmentate. Quando meperidina e promazina esseva administrate insimul, ni le consumo de oxygeno, ni le frequentia cardiac, ni le frequentia respiratori esseva alterate significativemente.

Promethazina produceva un notabile augmento del tension de sanguine quatro horas post le trauma. Le frequentia respiratori esseva etiam augmentate ante le traumatisation. Nulle de iste functiones esseva significative al nivello de un pro cento quando meperidina e promethazina esseva administrate concomitantemente, ben que un simile tendentia esseva apparente in ambe casos.

Miyagawanellosis of Cattle in Japan

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LYMPHOGRANULOMA venereum in man has been known in Japan for many years. However, other members of the genus Miyagawanella, *i.e.*, the psittacosis-lymphogranuloma (PL) group of viruses, have not been recognized until recently. The recent studies of miyagawanellosis in Japan were initiated by the etiologic study of a nonbacterial pneumonia of goats which has occurred epizootically since 1950 in Nagano, Kochi, and other prefectures. Studies [22,30] revealed that this disease was caused by a member of Miyagawanella which we designated "goat pneumonitis virus." [11,21,22] In the meantime, a similar virus of Miyagawanella was isolated from sick cattle.

Subsequent studies extended our knowledge concerning various aspects of miyagawanellosis of cattle in Japan and the properties of the causative agent, designated tentatively as "bovine PL virus." [16]

The studies of miyagawanellosis in cattle and goats were extended to include other species and resulted in discoveries of psittacosis in various avian species and human beings.[15,35] Recent study has revealed a virus of Miyagawanella in sheep.[10]

This report is intended to describe, in a composite form, the results of the studies of this disease in cattle in Japan. Most of the material presented herein has been published, but some of our hitherto unpublished data is included, also.

BRIEF HISTORY OF STUDIES

Our studies of miyagawanellosis in cattle were begun during an investigation of an epizootic bovine disease known as "bovine epizootic fever" or "bovine influenza" which swept over the western half of Japan in 1949 and 1950. The disease was characterized by fever, nasal discharge, salivation, dyspnea, and decreased lactation. Deaths were rare. Kiuchi and Inaba [12] and others isolated in mice and embryonating chicken eggs a number of viral strains from affected cattle. Later, some of those strains were identified as members of Miyagawanella.[29] Other groups of research workers, however, discovered that the etiologic agent of this disease was a virus different from those viral strains isolated by Kiuchi, Inaba, and others (*see* Omori *et al.*[24]). Furthermore, a relatively high incidence of complement-fixing antibodies to this virus of Miyagawanella suggested wide dissemination among cattle.[8]

Then, in May and June, 1953, a small epizootic of an unknown disease occurred among cattle in Shizuoka Prefecture. This disease was similar to sporadic bovine encephalomyelitis in the United States.[19,33] The etiologic agent was isolated from a naturally infected cow and identified as a member of Miyagawanella.[24,25]

Since 1953, a number of milking cows of Holstein-Friesian and Jersey breeds have been imported from North America, Australia, and New Zealand. A respiratory disease, characterized by fever, cough, and nasal discharge, was observed among these animals either aboard ship or at the quarantine station in Japan. Histopathologically, the disease was lobular or sublobar bronchopneumonia. Matumoto *et al.*[14] isolated the etiologic agent from nasal discharges, tracheal exudate, and tissue taken from lung lesions. This agent was identified as a member of Miyagawanella, also. Essentially the same results were obtained in investigations of indigenous cattle suffering from a similar respiratory disease.[4,7]

Some of the naturally infected cattle showing respiratory or nervous signs developed diarrhea, also. In some cases, signs of alimentary tract involvement were observed with little respiratory involvement, indicating alimentary tract infection with this virus. The virus was isolated from feces and intestines of sick cattle.[11,16]

These viral strains of bovine origin have essentially the same morphologic, serologic, and pathogenic properties for laboratory animals, *i.e.*, embryonating eggs, mice, guinea pigs, and other animals. These findings strongly indicate that they may be the same virus. We provisionally designated it "bovine PL virus." [16] In all probability, *Miyagawanella bovis* of York and Baker [28] and the virus of sporadic bovine encephalomyelitis of Wenner *et al.*[33] may be identical to our bovine PL virus, although further studies are necessary to reach a conclusion.[16]

A high incidence of fecal carriers was demonstrated among apparently healthy cattle. Furthermore, this condition persisted for long periods, providing a rich source of infective material for contamination of the environment. It is suggested that the virus excreted in feces from the infected hosts plays a major role in disseminating the virus among cattle, which may be infected by inhalation of dusts contaminated with infected feces or by ingestion of contaminated feeds.[16]

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Clinical Picture

The clinical course of the disease varied from inapparent to severe and involved mainly the central nervous, respiratory, and digestive systems. The clinical signs appeared in various combinations and degrees. Individual cases were classified on the basis of the most conspicuous signs.

The encephalomyelitic type of the disease is similar to sporadic bovine encephalomyelitis in the United States.[19] This type first attracted attention in a small epizootic which occurred in May and June, 1953, in Shizuoka Prefecture.[24] Since then, sporadic cases have been reported in various parts of Japan. A small number of cases was reported again in August, 1957, in Shizuoka Prefecture.

This type of disease was characterized by fever followed by paralysis of the limbs and other signs of central nervous system involvement. Usually signs of respiratory and alimentary system involvement were observed, also. During the acute phase, anorexia, depression, and lethargy occurred, followed by mild to severe diarrhea; the stools were mucous and apt to be watery and blue-gray. In some cases, nasal discharge, increased salivation, drooling, and rapid, labored respiration with associated cough appeared. Within a few days, the affected animal experienced difficulty in walking. Paralysis, mostly in the hindlegs, increased as the disease progressed. The animals staggered, circled, and fell. Heads were retracted in the position of opisthotonus. Finally, in severe cases, the affected animals, with increased paralysis, lay on the floor, unable to rise. Paralysis of the tongue and partial or complete loss of vision were often observed. Those cattle that were not affected severely usually recovered.

The pneumonic type was characterized by fever, nasal discharge, cough and, in some cases, diarrhea. There were mild cases, with slight nasal discharge and mild cough, and severe cases, with apparent pneumonic involvement. Usually the prognosis for the pneumonic type was good. Cases complicated by bacterial infection frequently terminated fatally.[14]

The intestinal type was characterized by fever and diarrhea only.[13] As mentioned previously, animals with encephalomyelitic and pneumonic types often had diarrhea as well.

In bovine PL virus infection, inapparent or latent infection was common. After recovery from clinical infection, many animals remained virus carriers, excreting the virus for a long time. Among apparently healthy cattle, there was a high incidence of fecal carriers.[16]

Pathologic Picture

At necropsy of animals suffering the encephalomyelitic type,[24] the central nervous system was free of gross changes except for an increase of cerebrospinal fluid and edema of the brain.

Histologically, hyperemia, small hemorrhages, aggregations of lymphocytes, and perivascular cellular infiltration were noticed in the meninges. General perivascular infiltration was seen throughout the gray and white matter of the brain and spinal cord (fig. 1). The thick perivascular cuffing consisted of swollen and multiplying cells accompanied by plasma cells and lymphocytes. Vacuolation and karyorrhexis of swollen large mononuclear cells and pyknosis of lymphocytes and plasma cells were often observed. There were few neutrophils. Multiplication of glia cells was generally seen in the brain and spinal cord, and small glial nodules composed mainly of microglial cells were seen. In some areas, there were nodular aggregations of erythrocytes. Pseudoneuronophagia was seen, but true neuronophagia was not observed. Some ganglion cells were swollen and had vacuolated nuclei.

Other conditions included congested and edema of the lungs, lobular pneumonia, small cellular nodules in the liver, and slight sinus catarrh of the lymph nodes.

Calves inoculated intracerebrally showed severe inflammation of the meninges in the early stages of the disease. In the later stages, the histologic picture was similar to that observed in animals with the natural infection.[24]

In sporadic bovine encephalomyelitis in the United States, fibrinous peritonitis, pleuritis, and pericarditis have been reported as conspicuous pathologic changes.[19] No such changes have been found in Japan; however, it is highly conceivable that they may occur here, also, considering that we performed necropsy on only a few cattle with natural infection. This is also indicated by the fact that experimental inoculation of cattle with the virus isolated in this country can produce serositis similar to that observed in sporadic bovine encephalomyelitis.

In the pneumonic type,[14] the lungs presented macroscopically lobular or sublobar lesions, mainly

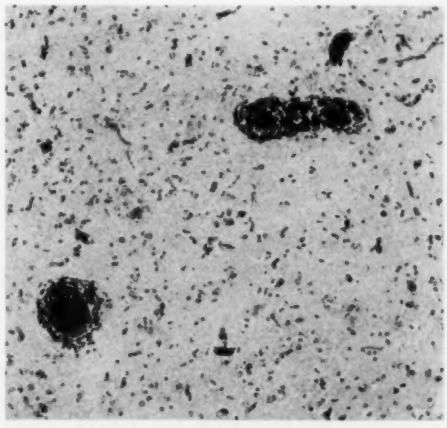

Fig. 1—Perivascular cuffing and glial infiltration in the corpus striatum of a naturally infected cow. H & E stain; x 130.

in the apical, medial, and intermedial lobes, particularly in the intermedial lobe. The lesions were light purplish red, grayish red, or grayish white, and in some parts showed mosaic patterns with patchy, apparently healthy areas within the lesions (fig. 2). Sectioned surfaces of the lesions had a reddish tint and were edematous. The color changed from red to gray and the lesions became granular, swollen, and then more friable. Air content was much reduced in the lesions. When complicated by bacterial infection, numerous large and small purulent lesions were observed along with the lesions due to the virus infection. Medullary swelling was noticed in the lymph nodes of the mediastinum and hilus of the lungs. Histologic examination of the lung revealed bronchitis, bronchiolitis, peribronchitis, and intraalveolitis. Changes in the alveoli consisted of accumulation of neutrophil and large mononuclear cells and serous exudate with fibrin nets (fig. 3). Interalveolitis was rarely observed.[14]

Cytoplasmic inclusion bodies were seen in epithelial cells of bronchioli in 1 animal with natural infection (fig. 4).[14]

EXPERIMENTAL INFECTION OF LABORATORY ANIMALS

Like other members of Miyagawanella, the virus multiplied well in embryonating chicken eggs. The virus inoculated into the yolk sac killed the chicken embryo in three to 11 days. Among the tissues and organs of embryonating eggs, the yolk sac had the highest affinity for the virus. Maximum infective titers of 10^{-7} to 10^{-8} (titrated in embryonating eggs) were reached in the yolk sac. However, the embryo and chorioallantoic membrane showed much lower titers, with allantoic and amniotic fluids having still lower titers.[25]

Fig. 2—The lungs of a calf 14 days after intratracheal inoculation with the 1485 strain of bovine PL virus. Arrows show lesions.

The virus had low pathogenicity for mice.[11, 25] Subcutaneous, intraperitoneal, or intracerebral inoculation had little effect on mice. However, the virus readily infected mice when intranasally instilled, as all the members of Miyagawanella do. When given intravenously, it produced little toxicity in mice.[11, 25] The low pathogenicity for mice made it possible to differentiate this virus from those of psittacosis, ornithosis, meningopneumonitis,[3] Illinois pneumonitis,[37] Louisiana pneumonitis,[13] and lymphogranuloma venereum, which can infect mice when given by intracerebral or intraperitoneal route. In regard to this property, bovine PL virus was similar to the bovine strains of York *et al.*,[36] and Wenner *et al.*,[33] goat pneumonitis virus,[21, 22] sheep abortion virus,[2, 17, 32] and feline pneumonitis virus.[1] Among the viruses derived from mammals, mouse pneumonitis and opossum viruses are different in this respect; they readily infect mice by intraperitoneal and intracerebral routes.[20]

In contrast with the low pathogenicity for mice, the virus had relatively high pathogenicity for guinea pigs.[11, 25] Thus, this virus readily infected guinea pigs by the intranasal, intraperitoneal, intracerebral, or subcutaneous route. The intraperitoneal route was particularly effective and produced fever, loss of weight, peritonitis, and swelling of the spleen, although a relatively large amount of virus was necessary to kill animals. Intraperitoneal inoculation produced complement-fixing antibodies in guinea pigs with a dose as small as 0.5 ml. of 10^{-6} dilution of infected yolk sacs.[10] Intranasal inoculation regularly produced lung consolidation. The high pathogenicity for guinea pigs is a property common to all strains derived from mammals, except mouse pneumonitis virus.[20] Strains of avian origin have little effect on guinea pigs, except the strains derived from turkeys[5] and egrets[28, 29] which are highly pathogenic for guinea pigs. Among the strains of human origin, Louisiana pneumonitis virus[13] is highly pathogenic for guinea pigs. However, the previously mentioned strains derived from man, turkeys, and egrets are differentiated from bovine PL virus by the fact that the latter has a lower pathogenicity for mice.

On the basis of pathogenicity for mice and guinea pigs, bovine PL virus appears to be closely related to the bovine strains of York *et al.*[36] and Wenner *et al.*,[33] goat pneumonitis virus,[22] sheep pneumonitis virus,[18] sheep abortion virus,[2, 17] and feline pneumonitis virus.[1] Among these strains, goat pneumonitis virus is more pathogenic for guinea pigs than bovine PL virus.[21] Thus, the virus from goats can kill guinea pigs with much smaller doses of infected yolk sacs, when inoculated intraperitoneally, and can produce more severe peritonitis than bovine PL virus. However, goat pneumonitis virus usually does not produce swelling of the spleen, a condition regularly observed in infection with bovine PL virus.[21] Our unpublished data show that sheep pneumonitis

virus [18] is similar to goat pneumonitis virus with respect to pathogenicity for guinea pigs. Feline pneumonitis virus seems to be less pathogenic for guinea pigs than bovine PL virus. The bovine strains of York *et al.* and Wenner *et al.* are most closely related to bovine PL virus. It is noteworthy that the virus isolated from sheep in Japan [10] is similar to bovine PL virus in pathogenicity for mice and guinea pigs.

In swine, bovine PL virus produced mild illness with fever, inactivity, and reduced appetite when given intracerebrally and pneumonitis when given intratracheally.[25] The virus also produced pneumonitis in goats when inoculated intratracheally.[11, 25]

THE EXPERIMENTAL DISEASE IN CATTLE

Three strains of bovine PL virus were inoculated into a number of cattle to demonstrate the etiologic relationship of these isolates to the natural disease observed in cattle. Of 20 cattle used, of Jersey and Holstein-Friesian breeds, 18 were 2 weeks to 6 months of age and 2 were 2 years old. The three strains were selected as representative of a large number isolated from (*a*) feces, (*b*) pulmonary tissue, and (*c*) central nervous system tissue of cattle with intestinal, pneumonic, and encephalomyelitic signs, respectively.

All the strains employed could reproduce all the types of the disease observed in natural case.

Thus, intratracheal inoculation produced typical pneumonia similar to that observed in cattle with the natural infection. Histopathologic findings in these cattle were consistent with those observed in cattle with natural infection. Even cattle which looked healthy, but actually were found to be infected by positive complement-fixation and isolation of the virus from feces, developed clinical pneumonitis after intratracheal inoculation with the virus.

Intracerebral inoculation with the virus produced signs of encephalomyelitis as well as systemic effects. In some cattle clinical signs referable to the central nervous system were relatively mild, but histopathologic examination revealed definite changes of encephalomyelitis and meningitis, and the virus was recovered from central nervous system tissues. Although the number of experimental animals was small, it was observed that cattle from which serum contained demonstrable complement-fixing antibodies before the experiment had a longer incubation period than those possessing no demonstrable antibodies.

Intraperitoneal inoculation produced fever, weakness, and anorexia, but no signs of either respiratory or nervous involvement. Fibrinous peritonitis was observed with all of the strains, but in only 4 of 9 cattle inoculated intraperitoneally. This behavior contrasted with that of sporadic bovine encephalomyelitis.[24] Failure to produce peritonitis in all animals inoculated by the intraperitoneal route may be explained on the basis of the widespread dissemination of the virus among Japanese cattle, making it impossible for us to obtain for the experiment cattle from herds free of infection

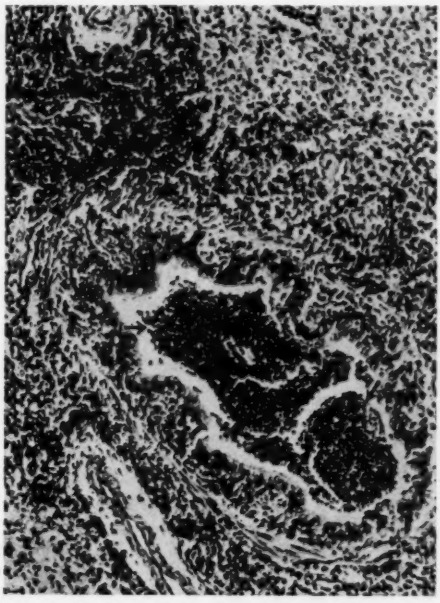

Fig. 3—Section of the lung of a calf showing peribronchitis and bronchiolitis with marked cuffing of mononuclear cells around a bronchiolus which has exudation with neutrophils in its lumen (arrow). H & E stain; x 100.

with the virus. This was illustrated by preinoculation isolation of the same virus from the feces of 9 of the 20 cattle utilized. It is noteworthy, however, that 3 of 4 cattle with a negative complement-fixation reaction before inoculation developed peritonitis, whereas only 1 of 5 with positive complement-fixation titer developed peritonitis. This finding may be further confirmation of the findings of Wenner *et al.*[23] that immunized cattle showed little change after intraperitoneal inoculation with their virus. Although this point has not been made fully clear, we suggest that the production of a fibrinous peritonitis depends more upon the state of immunity of the animal than upon differences between virus strains.

We were able to recover the virus from the fetus of a cow injected intravenously and killed 14 days later, despite her failure to develop signs of infection. This may indicate the possibility of abortion in naturally infected cows. Schoop *et al.*[21] have recently reported a virus of Miyagawanella as the cause of bovine abortion. Their virus, however, seems to be different in pathogenicity for mice from our bovine PL virus.

Endometritis (fig. 5) and mastitis were produced in cattle by inoculation of the virus into the uterus and into the mammary gland. It is not known if infection occurs in this manner naturally.

TABLE 1—Demonstration of Bovine Psittacosis-Lymphogranuloma Virus in Various Materials of Naturally Infected Cattle

Year (Reference)	Place	Breed	Clinical and pathologic findings	No. animals tested	Material tested	No. materials tested	No. materials virus positive
1953 Omori *et al.*[10]	Shizuoka Pref.	Native	Encephalomyelitis	1	CNS tissue	1	1
1953 Kawakami *et al.*[4]	Yokohama quarantine station	Jersey imported from U.S.A.	Pneumonia and mild dearrhea	2	Blood	2	0
					Nasal discharge	2	0
					Feces	1	1
1953 Kawakami *et al.*[4]	Yokohama quarantine station	Jersey imported from Australia	Diarrhea	2	Blood	2	0
					Feces	2	1
1954 Matumoto *et al.*[16]	Yokohama quarantine station	Jersey imported from U.S.A.	Pneumonia	5	Lung	5	1
					Nasal discharge	1	1
					Tracheal exudate	2	1
					Liver and spleen	1	0
					Intestine	1	0
					Mediastinal lymph node	1	1
					Feces	2	1
1954 Matumoto *et al.*[16]	Yamanashi Pref.	Holstein-Friesian	Healthy—pneumonia prevalent in area	6	Feces	6	2
1954 Matumoto *et al.*[16]	Yokohama quarantine station	Jersey imported from U.S.A., Australia, and New Zealand	Healthy—pneumonia prevalent in area	8	Feces	8	7
1954 Matumoto *et al.*[16]	Kanagawa Pref.	Holstein-Friesian	Healthy	16	Feces	14	7
					Nasal swab	15	0
					Milk	4	0
1954 Matumoto *et al.*[16]	Tokyo Pref.	Holstein-Friesian	Healthy	15	Feces	15	5
					Nasal swab	15	0
					Milk	14	0
1954–1955 Matumoto *et al.*[16]	Tokyo Pref.	Holstein-Friesian	Healthy	4	Feces	4	4

PATHOGENESIS OF THE DISEASE IN CATTLE

Our observations of the natural disease and the experimental disease in cattle revealed that it is a systemic infection. Encephalomyelitis or pneumonitis is incidental to or part of the systemic infection in which every organ system is potentially in danger of attack by the infectious agent. Incidence of pathologic lesions show that widespread distribution of the virus occurs in affected cattle.

The virus was demonstrated in various tissues and exudates obtained from naturally infected cattle (table 1). Thus, the virus was detected in the central nervous system of a cow affected with encephalomyelitis [24] and in the lungs, mediastinal lymph nodes, nasal discharges, and tracheal exudates of cattle affected with pneumonia.[4, 14] The virus was found also in feces of cattle suffering from pneumonia and diarrhea. [11, 14] A high incidence of the virus in feces was found among apparently healthy cattle.[16] In cattle experimentally inoculated by various routes, the virus appeared in feces. The virus was also detected in blood obtained during the acute phase in 3 of 8 cattle tested. In four of six attempts, the virus was isolated from the brain, spinal cord, or both of cattle inoculated intracerebrally. No virus was recovered, however, from the central nervous systems of 14 cattle inoculated by extraneural routes. Each of 3 cattle inoculated intratracheally carried the virus in the lungs; however, the virus was present also in the lungs in 8 of 18 cattle inoculated by intracerebral, intraperitoneal, or intravenous route. The virus was present in the liver in 4, spleen in 4, and kidney in 3 of 20 cattle inoculated by various routes. Of animals inoculated intracerebrally, the virus was isolated from the suprarenal gland in 1 of 6 tested and from mesenteric lymph node in 5 of 19 tested. Among cattle inoculated intraperitoneally, the virus was recovered from the great omentum in 2 of 3, from ascitic fluid in 1 of 4, and from intestinal tissue in 2 of 3 animals tested for virus.

On of the most distinguishing features of the virus is its tendency to persist for long periods in convalescent or in apparently normal, but infected, hosts. This ensures a large number of carriers and widespread dissemination of the infection (table 1).[15]

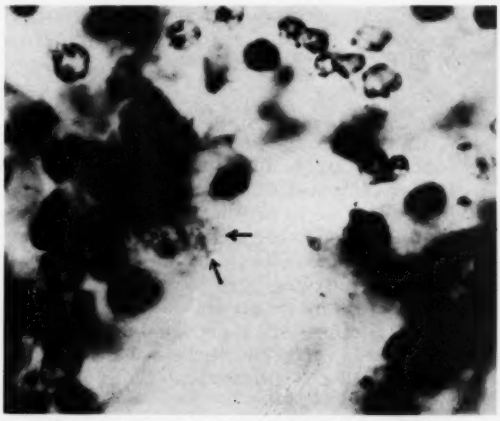

Fig. 4—A cytoplasmic inclusion body (arrows) in an epithelial cell of a bronchiolus in an animal with natural infection. H & E stain; x 900.

In bovine pneumonia due to this virus, secondary infection with pathogenic bacteria, such as Streptococcus, Hemophilus, and others was not rare. In such cases, the disease was severe and sometimes fatal. This disease often occurred during or immediately after transportation of animals. Pneumonia in cattle, occurring in relation to shipping of animals, is known as "shipping fever." Various investigators have isolated Streptococcus, Pasteurella, Hemophilus, Corynebacterium, and other bacterial species from the lungs of animals with

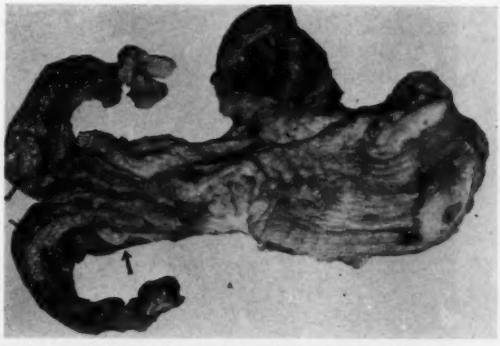

Fig. 5—Endometritis and vaginitis six days after inoculation of the virus into the uterine cavity of a cow with the Yamanashi No. 1 strain of bovine PL virus. Arrows show exudation with neutrophils.

this disease. It may be that bovine PL virus plays an important role in shipping fever in cattle.[14]

PROPERTIES OF VIRUS

Minute coccic elementary bodies were found readily in infected yolk sac smears. It was comparatively difficult to find them in exudate or impression smears of tissues obtained from experimentally infected mammals. The elementary bodies are tinctorially demonstrable with basic dyes and are seen readily under an ordinary light microscope. Both Giemsa's and Macchiavello's stains were used. The virus is characterized by formation of intracytoplasmic inclusion bodies (fig. 4).

Electron microscopy demonstrated spherical particles, 300–400 mμ in diameter, with a dense, somewhat irregular, central area.[14,24] Virus particles as large as 700 mμ in diameter are found rarely (fig. 6).

The virus passes through Berkefeld N and W filters. Infected yolk, yolk sacs, and their crude, heavy suspensions, stored at 4 C., retained infectivity for at least a month. Stored at −70 C., the virus remained active for more than a year.

Tetracycline, chlortetracycline, and oxytetracycline were effective in inhibiting multiplication of the virus in embryonating eggs. Erythromycin was effective also; penicillin and chloramphenicol were less effective, and streptomycin and sulfonamide compounds were of no demonstrable value.

IMMUNOLOGY

Boiled antigens[24] or ether-extracted antigens[24] prepared from infected yolk sacs fixed complement with serums of guinea pigs immunized by inoculation with bovine PL virus. No antigenic difference was discerned between strains of bovine PL virus by the complement-fixation reaction. Bovine PL virus showed a strong group relationship with the other members of Miyagawanella, *i.e.*, the viruses of goat pneumonitis, feline pneumonitis, and meningopneumonitis.[10,14,16,24]

Cattle artificially infected with the virus produced complement-fixing antibodies.[24] Complement-fixing antibodies were produced in guinea pigs by intraperitoneal inoculation of the virus even in small amounts. The infective titer of the virus, determined by the production of complement-fixing antibodies in inoculated guinea pigs, was as high as the titer based on the deaths of chicken embryos after virus inoculation into the yolk sac.[16] In demonstrating the virus in various bovine materials, the production of complement-fixing antibodies in guinea pigs inoculated with those materials is one useful criterion. This procedure is sufficiently simple to permit its use in large-scale survey.[16]

Immune serums of bovine PL virus inhibited hemagglutination of mouse erythrocytes by psittacosis virus.[6]

Although observations of immunity after infection in cattle are limited, it seems that there may be some resistance to a second

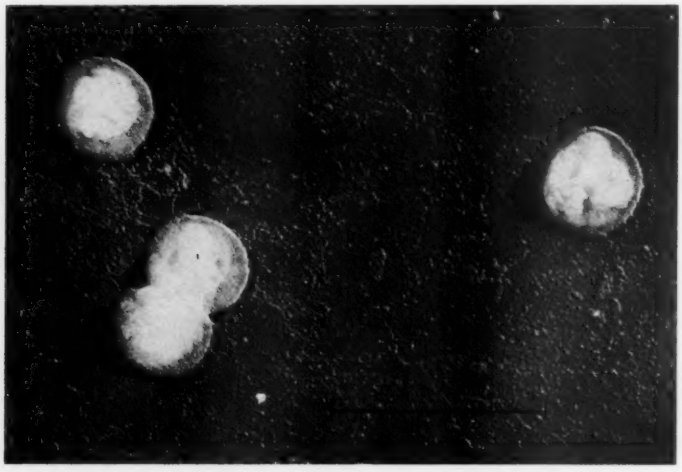

Fig. 6—Electron micrograph of elementary bodies of the Shizuoka strain of bovine PL virus. Chromium shadowing; x 30,000.

TABLE 2—Complement-Fixing Antibody for Bovine Psittacosis-Lymphogranuloma Virus Among Cattle in Japan (Nov. 1954-Sept. 1955)

District	No. of serums tested	Positive (No.) (%)	Equivocal (No.) (%)	Negative (No.) (%)	Anticomplementary (No.) (%)
Hokkaido	74	36 (48.6)	1 (1.4)	37 (50.0)	0
Tohoku	84	44 (52.4)	5 (6.0)	32 (38.1)	3 (3.5)
Kanto	114	46 (40.4)	6 (5.3)	41 (36.0)	21 (18.4)
Chubu	101	35 (34.7)	10 (9.9)	38 (37.6)	18 (17.8)
Kinki	48	11 (22.9)	0	37 (77.1)	0
Chugoku	104	20 (19.2)	3 (2.9)	81 (77.9)	0
Shikoku	20	7 (35.0)	1 (5.0)	12 (60.0)	0
Kyushu	32	3 (9.4)	1 (3.1)	28 (87.5)	0
Total	577	202 (35.0)	27 (4.7)	306 (53.0)	42 (7.3)

Note: Complement-dilution method [10] was employed: titers ≥ 1.2 are taken as positive, $1.1 \sim 0.8$ are taken as equivocal, ≤ 0.7 are taken as negative.

attack. In guinea pigs, resistance to infection following intracerebral or intraperitoneal challenge developed as a result of earlier infection with the virus. This immunization test in guinea pigs did not demonstrate any immunologic differences between bovine PL virus and goat pneumonitis virus. Bovine PL virus was recovered with relative ease from feces of both complement-fixation positive and negative cattle in the enzootic area.[16]

EPIZOOTIOLOGY OF THE DISEASE

A high incidence of complement-fixing antibodies indicates widespread dissemination of this virus or closely related virus[es] among cattle in Japan (table 2). This has been further attested by virus isolation from bovine materials. As shown (table 1), the virus has been demonstrated in bovine materials collected in Tokyo, Kanagawa, Shizuoka, and Yamanashi prefectures. Other investigators isolated the virus from cattle in Kyushu [12] and at the Akao branch of our institute where cattle were recruited from Shimane, Tottori, and Kyoto prefectures.[4]

Evidence has been obtained that the virus is distributed among cattle in other countries, also. The virus was isolated from cattle imported from the United States, Australia, and New Zealand (table 1).[11,14] There is no doubt that the virus was carried by the cattle from the countries of their origin. Bovine encephalomyelitis caused by this virus in Japan is similar to sporadic bovine encephalomyelitis reported by Menges *et al.* [19] and Wenner *et al.*[33] in the United States, and *Miyagawanella bovis* isolated by York and Baker [36] from the intestines and feces of apparently normal calves in the United States is similar to bovine PL virus.

Clinical infections occur sporadically or in epizootics. Sometimes the disease occurs during transportation of animals or soon after their arrival at their destination.

Epizootiologically, it is important to know the sites from which the virus leaves the infected host. The virus has been isolated from nasal or tracheal secretions of cattle suffering from pneumonia,[4,14] while nasal secretions of clinically healthy cows have been tested with completely negative results.[15] On the other hand, a high incidence of the virus in feces has been found, not only among clinically sick cattle, but also among apparently healthy cattle.[16] Furthermore, the presence of virus in the feces continued for a long time although the infective titer of feces was not high, usually in the range of 10^{-1} to 10^{-2}. In one case, the virus was recovered from feces of an animal one year after the first positive isolation.

When mastitis and vagino-endometritis were produced in cows by experimental inoculation, the virus was recovered from the milk and from secretions of the uterus and vagina. This indicates that the infection may be spread from animal to animal by means of these secretions. However, our limited investigations have not confirmed this.[16]

The most likely means of virus transmission from animal to animal seems to be the feces. Susceptible hosts may acquire infection by inhalation of dust contaminated with infected feces or by ingestion of contaminated materials. Similar findings regarding transmission have been obtained in studies with goat pneumonitis virus.[26]

DIAGNOSIS

The diagnosis of this disease is difficult because of the variability of clinical signs

and the wide distribution of latent infection. The clinical signs alone do not provide sufficient information for establishing a diagnosis. Complement-fixation tests, virus isolation, or both are necessary for establishing a definite diagnosis. In fatal cases, pathologic and histologic examinations aid in diagnosis.

For virus isolation, nasal washings and blood may be employed; in fatal cases, organ tissues can be used (table 1). The preferred test animal is the guinea pig. To suppress bacterial contamination, streptomycin is used in a concentration of 5 to 10 mg. per milliliter. Guinea pigs are inoculated intraperitoneally with 1 to 3 ml. of the material and killed five to seven days following inoculation. The liver and spleen are harvested, and a 10 per cent suspension of those organs, after addition of streptomycin, is inoculated into 7-day-old embryonating eggs by the yolk sac route. When the virus is present, chicken embryos will die in five to ten days, and elementary bodies characteristic of Miyagawanella will be shown in smears of the yolk sacs stained by Giemsa's or Macchiavello's methods. Further identification is accomplished by complement-fixation and pathogenicity tests in experimental animals.

Routinely, production of complement-fixing antibodies in the guinea pigs inoculated with the test material may be substituted for the actual isolation of the virus. When this is done, the guinea pigs are bled about four weeks after inoculation.

If the virus is isolated from nasal washings or from lung lesions of cattle showing respiratory involvement, the diagnosis is positive. In the case of encephalomyelitic type, the virus may be isolated from the central nervous system. In complement-fixation tests, it is necessary to show a significant increase in titer between serum specimens of the acute and convalescent stages.

TREATMENT AND CONTROL MEASURES

Since the tetracycline group of antibiotics and erythromycin effectively inhibit multiplication of the virus in embryonating eggs, these antibiotics may be useful in treatment of affected cattle. Tetracycline has been used effectively to treat pneumonia produced by intratracheal inoculation with goat pneumonitis virus in goats.[9] However, its cost may limit its practicability.

In control of the disease, the widespread latent infection of cattle in enzootic areas is of great importance. Eradication of pneumonitis virus from goat feces is effected by administration of tetracycline.[27] This procedure may be applicable also to infection of bovine PL virus in cattle, if it is not too costly.

SUMMARY

The most prominent features of infection of Japanese cattle by a member of the genus Miyagawanella, the psittacosis-lymphogranuloma (PL) group of viruses, have been presented. Accumulated data concerning the clinical picture of the natural disease in cattle and the experimental disease in cattle and laboratory animals are recorded. The properties of the virus, indicated means of transmission, and diagnosis, treatment, and control measures for the disease are reported.

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SUMMARIO IN INTERLINGUA

Miyagawanellosis de Bestial in Japon

Es presentate le plus prominente aspectos del infection de bestial in Japon per microorganismos del genere Miyagawanella (le gruppo de virus de psittacosis-lymphogranuloma, designate per PL in iste articulo). Es summarisate le datos accumulate con respecto al quadro clinic del morbo natural in bestial e a formas experimental del morbo in bestial e animales de laboratorio. Le proprietates del virus, le cognoscentias currente relative al transmission de illo, e le diagnose, le tractamento, e le mesuras de subjugation del morbo es discutite.

A Comparison of a Hemagglutination Test with a Modified Hemolytic Test on Serums from Intradermal Bovine Tuberculin Reactors and Nontuberculous Cattle

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THE INTRADERMAL tuberculin test has proved to be the most reliable aid in the diagnosis and eradication of bovine tuberculosis, but it has long been recognized that routine intradermal tests have certain limitations.[10,27,28]

A number of serologic tests have been applied to the diagnosis of tuberculosis in man and animals, but none has proved reliable enough for routine use. The agglutination by specific antiserums of erythrocytes coated with bacterial polysaccharides was reported by Keogh *et al.*[11,12] In 1948, Middlebrook and Dubos[16] described a hemagglutination test which could detect specific antibodies in the serums of tuberculous cattle and human beings. In this test they employed a new type of antigen consisting of washed normal sheep erythrocytes sensitized by adsorption with the water-soluble extract of human tubercle bacilli. Scott and Smith[28] modified this method by substituting old tuberculin (four times the strength of standard tuberculin) for the aqueous extract of human tubercle bacilli as the sensitizing agent for sheep erythrocytes. Rothbard *et al.*[20] reported confirmation of this test. Sohier *et al.*[25] reported satisfactory results with this test using human O red blood cells sensitized with powdered tuberculin designated as IP 48. Larsen *et al.*[14] described a hemagglutination test for the diagnosis of paratuberculosis in which a purified protein derivative (PPD) of johnin was used to sensitize the sheep red blood cells. Kuttler[13] reported satisfactory results with the hemagglutination test on bovine serums using tuberculin PPD to sensitize sheep red blood cells.

In 1950, Fisher and Keogh,[5] Middlebrook,[15] and Fisher[3] described a hemolytic modification of the hemagglutination test. To the antigen and serum, they added complement that caused hemolysis instead of hemagglutination in serums containing antibodies against tuberculosis.

Additional papers[1, 2, 4, 6, 7, 9, 13, 18, 19, 21, 24, 26] have reported varied results using these tests or modifications of them in diagnosing tuberculosis in cattle. Mollov and Kott[17] and Schwartz *et al.*[22] made a comparison of the hemagglutination test and the hemolytic test in diagnosing tuberculosis in man and concluded that the hemolytic test was the most promising.

Each of 48 cattle that had reacted to the regular intradermal test one or two weeks previously were retested by Kuttler[13] with regular tuberculin, regular johnin, and four fractions of these acid-fast allergens. Blood samples were obtained immediately before and 72 hours after retest, and both hemagglutination and hemolytic reactions showed insignificant fluctuation in serologic titers. Kuttler came to the conclusion that the original tuberculin caudal fold test would not influence the serologic titers and that the test on tuberculin-reactor cattle before slaughter would represent a valid evaluation of the serologic techniques.

This paper shows a comparison of a hemagglutination test with a hemolytic test on serums from 1,410 intradermal tuberculin reactors and 232 nontuberculous cattle.

MATERIALS AND METHODS

During the period from Jan. 1, 1956, to Jan. 1, 1957, a hemagglutination test and a modified hemolytic test were performed on serums from 1,410 intradermal bovine tuberculin reactors and 232 nontuberculous cattle. Of the 1,410 intradermal bovine tuberculin reactors, 248 were from retests of herds that had intradermal tuberculin reactors to the first tuberculin test. All reactors to the first tuberculin test were slaughtered before retesting the herds after 30 to 90 days. The purpose of waiting at least 30 days between tests was to avoid desensitization. On the day the reactors were observed and branded, 10 ml. of blood was drawn from each intradermal reactor. The blood was allowed to clot, the serum drawn off and preserved with aqueous thimerosal* at a final dilution of 1:10,000, and mailed to this laboratory for testing. The Animal Disease Eradication Division furnished a copy of the report of necropsy on each intradermal tuberculin reactor. All intradermal reactors were slaughtered at a plant that had federal meat inspection. The nontuberculous serums were obtained from the

From the Regional Animal Disease Research Laboratory, Agricultural Research Service, USDA, Auburn, Ala.

The author thanks Dr. A. F. Ranney, head, Tuberculosis Eradication Section, Animal Disease Eradication Division, Agricultural Research Service, Washington, D.C., and his field force for supplying necropsy reports, herd histories, and serums from the tuberculin reactors; and Dr. Richard A. Damon, Biometrical Services, Agricultural Research Service, Beltsville, Md., for the statistical analysis of the data.

* Merthiolate, produced by Eli Lilly & Co., Indianapolis, Ind.

TABLE 1—Comparison of a Hemagglutination and a Modified Hemolytic Test on Serums from 1,410 Intradermic Tuberculin Reactors and 232 Nontuberculous Cattle

Test	78 (5.5%), generalized lesions		536 (38.0%), localized lesions		36 (2.5%), skin lesions		760 (53.9%), no gross lesions		232 nontuberculous animals	
	Positive (1:32 and above)	Susp. or neg. (1:16 or less)	Positive (1:32 and above)	Susp. or neg. (1:16 or less)	Positive (1:32 and above)	Susp. or neg. (1:16 or less)	Positive (1:32 and above)	Susp. or neg. (1:16 or less)	Positive (1:32 and above)	Susp. or neg. (1:16 or less)
Hemagglutination	23 (29.5%)	55 (70.5%)	91 (17.0%)	445 (83.0%)	4 (11.1%)	32 (88.9%)	48 (6.3%)	712 (93.7%)	1 (0.4%)	231 (99.6%)
Hemolytic	47 (60.3%)	31 (39.7%)	221 (41.2%)	315 (58.8%)	8 (22.2%)	28 (77.8%)	120 (15.8%)	640 (84.2%)	3 (1.3%)	229 (98.7%)
	$X^2 = 15.299$*		$X^2 = 76.440$*		$X^2 = 3.294$		$X^2 = 40.052$*		$X^2 = 1.039$	

* Significant at 1 per cent.

Alabama Brucellosis Laboratory. The 232 serums from nontuberculous cattle were from ten dairy herds selected by a federal veterinarian as being free from tuberculosis infection. There had been no previous history of tuberculosis infection, and all herd replacements were raised on the farm.

Preparation of the Serum, Erythrocytes, and Antigen.—These preparations were similar to those described by Larsen *et al.*,[11] except that the PPD used was prepared from culture filtrates of *Mycobacterium tuberculosis* var. *hominis* by the Weybridge method.[8] Also, the final dilution of antigen was a 0.25 per cent suspension of sensitized cells that, when hemolyzed, had an optical density of 0.30 ± 0.01 when measured with equal parts of distilled water at 580 mμ in a Bausch and Lomb Spectronic 20 colorimeter.

The Hemagglutination and Hemolytic Tests.—The hemagglutination test procedure described by Larsen *et al.*[11] was used, except that a 0.25 per cent suspension of antigen was employed instead of 0.2 per cent suspension. The hemolytic test was performed the same way, on the same day, and with the same materials as those used in the hemagglutination test, except that after mixing the serum and antigen, the tubes were placed in a refrigerator for two hours and, upon removal from the refrigerator, 1 drop of complement (4 units) was added to each tube. The reagents were shaken and incubated for one hour in a water bath at 37 C., then placed in a refrigerator (approximately 8 C.) and read the following morning. Any tube that had 100 per cent hemolysis was considered positive.

Titration of Complement.—Complement titration was done by determining the minimum hemolytic dose of complement in the hemolytic system itself. A serum was used that gave a hemolytic titer of 1:128 with cells fully sensitized with tuberculin PPD in the presence of excess complement. Tubes containing 0.4 ml. of a 1:32 dilution (4 units) of the test serums and 0.4 ml. antigen were refrigerated for two hours at 8 C.; then 1 drop of 1:8, 1:10,1:12, 1:14, 1:16, 1:20, 1:24, 1:28, 1:32, or 1:40 dilution of complement was added to each tube and incubated in a water bath for one hour at 37 C. The highest dilution of complement that gave complete hemolysis was 1 minimum hemolytic dose (1 unit). In routine testing, 4 units of complement per tube was used. The complement was diluted so that 1 drop would contain 4 units. A commercial complement* made from fresh guinea pig serum dried from the frozen state was used in all the tests.

RESULTS AND DISCUSSION

The results obtained with the hemagglutination and the hemolytic tests on the 1,410 intradermal tuberculin reactors and 232 nontuberculous cattle are shown (table 1), grouped according to the type of lesions.

* Bacto-complement, produced by Difco Laboratories, Detroit, Mich.

The hemolytic test gave significantly more positive reactions than the hemagglutination test on serums from cattle showing generalized lesions, localized lesions, and no gross lesions (table 1). There was no significant difference in the two tests on serums from either the cattle having skin lesions or the nontuberculous cattle. Both serologic tests gave their highest proportion of positives in cattle with generalized lesions. Reactions in those having localized lesions, skin lesions, and no gross lesions followed in decreasing order.

There were higher serologic titers on serums from reactors to the first intradermal tuberculin test than there were on serums from reactors to the second intradermal test. These results show that 56.8 per cent of the serums from cattle found to have lesions following the first intradermal test were positive to the hemolytic test and 28.4 per cent were positive to the hemagglutination test. On the second intradermal test, 18.0 per cent of the serums from cattle with lesions were positive to the hemolytic test and none was positive to the hemagglutination test. The hemolytic test also gave more positive reactions than the hemagglutination test on the first and second intradermal test reactors that had no gross lesions. Of the reactors to the first intradermal test, 60.3 per cent had lesions at necropsy and 43.89 per cent of the reactors to the second intradermal test had lesions at necropsy.

A satisfactory serologic test should give positive titers for all adult cattle shown to have tuberculous lesions at necropsy. The results show that 43.6 per cent of the intradermal reactors with lesions were positive to the hemolytic test and 18.4 per cent were positive to the hemagglutination test.

If a 1:16 titer were used as positive, 54.9 per cent of the intradermal reactors with lesions would be positive to the hemolytic test and 35.7 per cent to the hemagglutination test; however, in normal animals, there would be approximately 7.0 per cent false positives to both the tests. These results are not shown in the table.

A low serum titer does not exclude tuberculosis; however, a high titer is a good indication of tuberculosis activity or closely related diseases, such as Johne's disease.

The hemolytic and hemagglutination tests would have to be improved to be of much value as possible diagnostic tools for case-finding in bovine tuberculosis.

SUMMARY

A comparison of a hemagglutination test with a modified hemolytic test on serums from intradermal bovine tuberculin reactors and nontuberculous cattle was made. The hemolytic test was superior to the hemagglutination test in detecting antibodies in serums from 1,410 intradermal bovine tuberculin reactors. In both tests, less than 7.0 per cent of the serums from 232 nontuberculous cattle had titers of 1:16 and above. However, as presently used, these tests were not as reliable as the intradermal tuberculin test since only 54.9 per cent of the tuberculin reactors with lesions had titers of 1:16 or above in the hemolytic test and only 35.7 per cent in the hemagglutination test. It was also found that the hemagglutination and hemolytic titers were higher on serums from cattle that reacted to the first tuberculin test than on serums from cattle in the same herd that reacted to the second tuberculin test.

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SUMMARIO IN INTERLINGUA

Comparation Inter Un Test de Hemagglutination e Un Modificate Test de Hemolyse con Seros ab (1) Reactores a Tuberculina Bovin Intradermal e (2) Bestial Nontuberculotic

Esseva effectuate un comparation inter un test de hemagglutination e un modificate test de hemolyse in seros ab reactores a tuberculina bovin intradermal e ab bestial nontuberculotic. Le test de hemolyse esseva superior al test de hemagglutination in deteger anticorpore in le seros de 1.410 reactores a tuberculina bovin intradermal. In ambe tests, minus que 7,0 pro cento del seros de 232 pecias de bestial habeva titros de 1:16 e plus. Tamen, in le forma in que iste tests es currentemente usate, illos non es tanto fidel como le test a tuberculina intradermal, proque solmente 54,9 pro cento del reactores a tuberculina con lesiones habeva titros de 1:16 o plus in le test de hemolyse e solmente 35,7 pro cento in le test de hemagglutination. Esseva etiam constatate que le titros de hemagglutination e de hemolyse esseva plus alte in le sero ab animales que reageva in le prime test a tuberculina que in le sero ab animales del mesme grege que reageva in le secunde test a tuberculina.

The Catalase, California, and Cell Count Tests for Detecting Abnormalities in Milk

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MANY DIAGNOSTIC TESTS for mastitis have been developed and recommended. Tests responding to inflammatory alterations of milk are cheaper and quicker than bacteriologic examinations and may be adequate for certain purposes. For dairy farmers, daily use of the strip cup is the most practical check on abnormalities due to mastitis. There is need for a more accurate simple barn test than the strip cup, and California workers have recently proposed a modification of the Whiteside test for this purpose. This is called the California mastitis test (CMT).[8] Breed counts of body cells are used widely in sanitary and public health laboratories. Alkalinity, curd tension, chloride concentration, and electrical conductivity have all been used as criteria of inflammatory abnormalities in milk. The catalase enzyme is found in milk as a result of mastitis and has been used as an indicator of abnormality.

The catalase test for mastitis is based on the decomposition of hydrogen peroxide to form gaseous oxygen by the catalase activity of abnormal milk.[3] Davis *et al.*[1] found such a poor correlation between catalase function and high cell counts of market milk that they considered the catalase test unreliable as an indicator of inflammatory products in market milk. Kastli and Binz[4] reported that cell numbers did not strictly parallel catalase content, but that there was a fairly good correlation in general. Proscholdt[7] demonstrated a relationship between the catalase test and cell content of milk, but encountered many discrepancies. Sommers[9] considered catalase content as a test for samples of milk from individual quarters and described several techniques of testing for catalase. Monlux[6] thought the catalase test to be a valuable diagnostic aid, although he found it unreliable in early or late lactation.

Detection of mastitis organisms by bacteriologic culture of quarter milk samples has been emphasized for control or therapy of the disease in recent years. Though undoubtedly of value, such bacteriologic tests are expensive and require aseptically collected individual milk samples. Furthermore, culture tests do not distinguish between carriers, animals with mild cases, and animals with severe cases, and are of little value when used on mixed herd milk. Bacteriologic tests on milk do not correlate well with cell counts, or with the Whiteside test.[2]

Milk sanitarians and dairy plant operators need a quantitative test for mastitis on mixed herd milk so they can enforce the elimination of grossly abnormal milk from the market supply. In most areas, herd samples for such tests can be collected from farm bulk tanks by truck operators.

This investigation was made to determine the reproducibility and correlation of several mastitis tests that might be used on mixed herd milk.

MATERIALS AND METHODS

Samples of mixed herd milk were collected in the vicinity of Madison, Wisconsin and Pullman, Washington. For body cell counts, 0.01 ml. of thoroughly mixed milk was transferred, by means of a 0.01 volumetric pipette, to a clean glass slide and spread with a needle over an area of 1 square centimeter, as determined by a ground glass plate in the background. Two smears were made from each milk sample. The smears were allowed to dry in the air and then were fixed by gentle heating. They were defatted by immersing for one minute in Newman's solvent. Smears were then stained by immersing the slides in 0.1 per cent aqueous methylene blue for one minute and washing in tap water. Body cells were counted in 50 successive fields under the oil immersion objective of the microscope. The number of body cells per milliliter were calculated by multiplying the field area factor of the microscope by the average number of cells per field.

The equipment and technique used for the cata-

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Part of this work was performed at the Wisconsin Agricultural Experiment Station by both authors and part by the senior author at Washington State College.

lase test were similar to that proposed by Merchant and Packer,[5] and are illustrated (fig. 1). Screw-capped test tubes, marked at a 10-ml. volume, were used in many of these experiments. Screw-capped centrifuge tubes of 15-ml. capacity were found to be satisfactory, and any test tube could be used if stoppers containing a small U tube were inserted.

Variations in the technique were tried to determine the optimum time and temperature of holding and the optimum concentrations of reagents for generation of gas.

Experimental Procedure and Results

The Optimum Quantity of Hydrogen Peroxide and the Rate of the Catalase Reaction.—Three tests on each of 11 samples of mixed herd milk were made using 0.5, 1.0, and 1.5 ml. of hydrogen peroxide U.S.P. (3%), respectively, in each test. The tests were held at 26 C. and the quantity of oxygen was measured at one-half, one, two, three, four and one-half, six, and 22 hours after mixing the reagents. The average results were recorded (fig. 2). The per cent of oxygen was greater with larger quantities of hydrogen peroxide, but there was only a slightly larger amount of oxygen in samples with 1.5 than with 1.0 ml. of hydrogen peroxide solution. These results were interpreted as meaning that there was little advantage, if any, in adding more than 1.0 ml. of hydrogen peroxide solution to 9 ml. of milk.

The reaction progressed rapidly and was nearly terminated within three hours after mixing the ingredients (fig. 2). The volume of oxygen in the tube remained relatively constant during the period from three to 22 hours.

The Effect of Temperature on the Catalase Test.—Replicate tests were carried out on 18 samples at refrigerator temperature (3 to 5 C.), at room temperature (26 to 28 C.), and at 37 C. The results were averaged (table 1). At 3 to 5 C., the reaction progressed more slowly and continued over a longer time, so the final quantity of oxygen was markedly larger than at room temperature. At 37 C., the reaction progressed rapidly at first, but the final volume of oxygen was smaller than at room temperature. When incubated at 3 to 5 C. for 21 hours, there were greater differences between samples with low and high catalase values than at room temperatures.

In another experiment, varying amounts of hydrogen peroxide were added to six samples at room and refrigerator temperatures. As in the previous experiment, larger volumes of oxygen resulted at refrigerator than at room temperature after 21 hours. Increasing the amount of hydrogen peroxide resulted in higher catalase values, but not in proportion to the amount of hydrogen peroxide added. As before, there appeared to be no advantage in adding more than 1 ml. of hydrogen peroxide to 9 ml. of milk at either refrigerator or room temperatures.

The Reproducibility and Correlation of the Catalase Test and Cell Count Results.—Ten replicate catalase tests were made on each of three samples of milk. Ten Breed

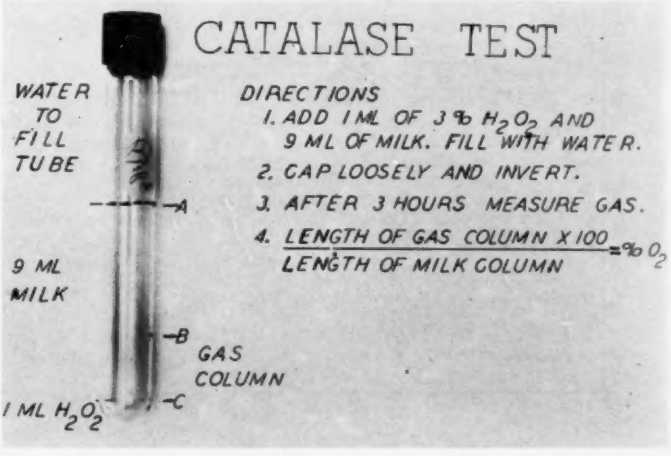
CATALASE TEST
WATER TO FILL TUBE
DIRECTIONS
1. ADD 1 ML OF 3% H_2O_2 AND 9 ML OF MILK. FILL WITH WATER.
2. CAP LOOSELY AND INVERT.
3. AFTER 3 HOURS MEASURE GAS.
4. LENGTH OF GAS COLUMN X 100 / LENGTH OF MILK COLUMN = % O_2
A
9 ML MILK
B
GAS COLUMN
C
1 ML H_2O_2

Fig. 1—Apparatus and technique for the catalase test.

TABLE 1—Effect of Temperature and Time on the Average Per Cent of Oxygen Evolved in the Catalase Test on 18 Samples of Mixed Herd Milk

Temperature (C.)	Time of reading (hr.)			
	2	4	5	21
3 to 5	6.9	15.8	18.5	34.8
26 to 28	18.3	22.5	23.3	.....
37	15.6	17.5	18.0	.....

smears were also made on each of these samples. This provided replicate tests of the same milk sample for a determination of the reliability of the cell count and catalase technique. The mean result and the standard deviation for both the catalase tests and cell counts were recorded (table 2). The catalase test values on a single sample were consistent, with a standard deviation from 3.5 to 5.0 per cent oxygen. Cell counts had a standard deviation of nine to 30 cells per 50 fields.

The catalase test and cell count results were compared on 163 mixed herd and individual quarter samples of milk. There appeared to be a fairly good correlation between the two tests, although a few individual samples with high catalase values had a low cell count. Thirty of 37 samples with cell counts above 1 million per milliliter produced more than 25 per cent oxygen in the catalase test.

The Effect of Delayed Testing.—Thirty-four mixed herd samples were taken at a dairy plant and catalase tests were started within two hours. The remainder of each sample was held at 26 to 28 C. for six hours, and at 3 to 5 C. for 16 hours, at which times it was tested again. The first test disclosed an average of 21.5 per cent oxygen, while the tests subsequent to holding disclosed 19.2 per cent oxygen. This indicated that a small proportion of the catalase (about 10%) was probably inactivated by the holding procedure. A moderate amount of bacterial growth had no effect.

The Distribution of Catalase Values of Milk from 138 Herds.—Fourteen (10%) of the herds produced milk with a catalase value greater than 40 per cent, and an additional 13 per cent yielded milk giving less than 40 but more than 30 per cent oxygen (fig. 3). An examination was made of quarter samples of fore-milk from cows in seven herds, utilizing the Hotis test, microscopic examination of incubated samples, and streak-plating of incubated samples on blood agar. The results of the catalase test on herd milk were compared to the results from individual quarter samples (table 3). Cata-

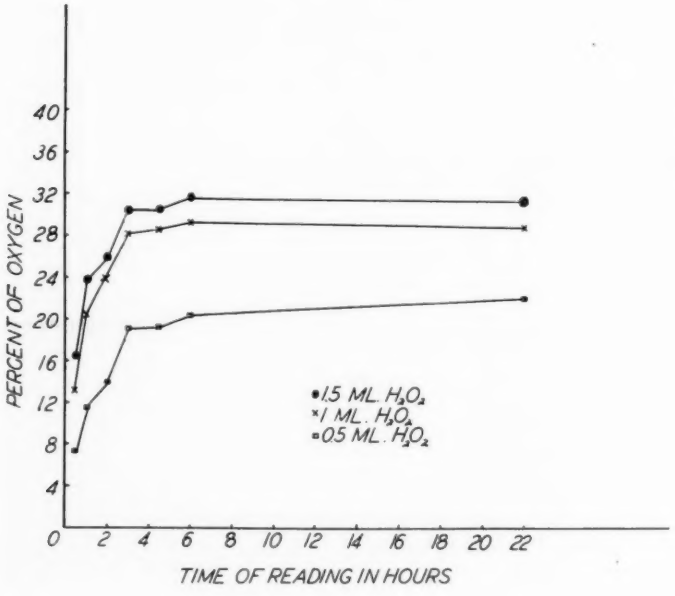

Fig. 2—The average effect of using varying amounts of hydrogen peroxide U.S.P. (3%) in catalase tests of samples of mixed herd milk from 11 different herds.

lase test results appeared to be more related to cell counts but somewhat related to the incidence of streptococcic infection. The cell counts were high in a large proportion of quarter samples in herds whose pooled milk yielded high catalase values. The cell count was not, however, a completely reliable criterion of the incidence of streptococcic infection.

Comparison of Tests.—Cell counts, catalase tests, and CMT were performed on 263 samples of fore-milk from 66 cows in a herd in which staphylococcal mastitis was the principal udder disease. The results were compared and correlation coefficients were calculated.

Most samples with a catalase result under 20 had low cell counts, whereas a few with higher catalase values also had low cell counts (table 4). The correlation coefficient between these tests is 0.52, but this figure may not be reliable because of the asymmetric distribution.

Numerous discrepancies are also found in the comparison between the catalase test and the CMT (table 5). Many samples with catalase reactions above 20 had negative, trace, or "1" reactions in the CMT. Only three samples with high CMT results (2 or 3) had low catalase values. The distribution of variability between these two tests was symmetrical and the correlation coefficient was 0.75.

The comparison between CMT and cell counts (table 6) reveals numerous discrepancies, although the correlation coefficient was 0.71. The broad distribution and conflicting results suggest that the CMT does not depend on the number of body cells for its entire reaction.

These 263 samples were cultured directly on Difco blood agar containing 6 per cent bovine blood and the organisms recovered were identified. In 94 samples negative to all three tests (cell count, <500,000, catalase <5%, CMT negative), 29 per cent contained hemolytic staphylococci. Six samples with very high values in all three tests were negative on culture, indicating failure of the culture to disclose the cause of the inflammatory reaction. No one or any combination of the three tests was a reliable indicator of the presence or absence of a staphylococcal infection.

Source and Nature of the Milk Component Causing Reactions.—Samples of milk were tested with both the CMT and catalase test, and 50-ml. portions were then centrifuged at 3,000 r.p.m. for 30 minutes. The top fluid (including the cream), the middle portion, and the sediment were removed and again subjected to the two tests. The results indicated that catalase was in much higher concentration in the sediment and in somewhat lesser concentration in the cream fraction. A lower, but appreciable, amount of catalase could be detected in the center portion which had no cells by microscopic examination. The reactions to the CMT were similar.

The quantity of blood in milk required to produce various catalase reactions was determined by adding increasing concentrations to samples of fresh milk which had been boiled to destroy all catalase. An appreciable reaction was produced by 0.05 per cent blood, and 0.5 to 1.0 per cent blood gave a maximal reaction with the methods used (fig. 4).

TABLE 2—The Reproductibility of the Catalase Test and the Breed Cell Count as Determined by Ten Individual Tests on Each of Three Samples of Mixed Herd Milk

	Catalase tests		Breed cell counts	
Sample	Mean % oxygen	s.d.*	Mean No. cells per 50 fields	s.d.*
27	8.03	3.5	86.1	14.6
47	20.75	4.91	93.6	8.9
51	30.95	5.03	242.5	30.0

* Standard deviation.

DISCUSSION

The catalase test, as described, is a simple procedure requiring materials readily available. According to the work done, the most practical technique involves the addition of 1 ml. of hydrogen peroxide U.S.P. to 9 ml.

TABLE 3—A Comparison of the Catalase Test on Mixed Milk from Herds with the Incidence of Streptococcic Infection and Body Cell Counts of Samples of Fore-Milk from Individual Quarters

			Per cent of quarters with cell counts		
Herd	Catalase value	Per cent of streptococcic infection	Less than 1,000,000	1,000,000 to 3,000,000	Over 3,000,000
H	12	16.7	63	25	12
S	19	34.3	79	10	11
F	32	40.6	73	7	20
R	32	61.7	84	8	8
Ho	36	40.9	40	26	34
Ha	39	48.9	77	4	19
W	54	38.5	20	18	62

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TABLE 4—Relationship of the Catalase Test to the Breed Count of Body Cells on Quarter Samples of Milk

Catalase % O_2	Cell count in thousands					
	0–500	501–1,000	1,001–1,500	1,501–2,000	2,000 and up	Totals
0–10	137	3	0	0	0	140
10.1–20	27	7	3	1	1	39
20.1–30	15	4	2	2	4	27
30.1–40	4	4	2	0	7	17
40.1 and up	5	4	1	1	29	40
Total	188	22	8	4	41	263

of milk and subsequent holding for three hours at room temperature. The temperature affects the rate of the reaction and, hence, excessive thermal variations should be avoided.

The accuracy of the catalase test as an indicator of abnormalities in milk caused by mastitis was definitely established. The test results were consistent in replicate trials on the same sample, as indicated by

TABLE 5—Relationship Between the Catalase Test and the California Mastitis Test on Quarter Samples of Milk

Catalase % O_2	California mastitis test					
	—	Tr	1	2	3	Totals
0–10	116	18	4	1	0	139
10.1–20	14	13	10	1	1	39
20.1–30	13	8	4	2	0	27
30.1–40	6	2	6	1	3	18
40.1 and up	0	5	6	17	12	40
Totals	149	46	30	22	16	263

a standard deviation in values of 3.5 to 5.0 per cent oxygen. Any test for mastitis abnormalities in mixed herd milk would be expected to vary according to the relative quantity of abnormal milk entering the supply. Samples from individual cows presumably would be more accurate for testing than samples from the combined milk of several cows.

TABLE 6—Relationship of the Breed Count of Body Cells to the California Mastitis Test on Quarter Milk Samples

Body cells (thousands)	California mastitis test					
	—	Tr	1	2	3	Totals
0–500	141	29	14	4	3	188
501–1,000	4	3	11	3	1	22
1,001–1,500	1	6	0	0	1	8
1,501–2,000	0	3	2	0	0	5
2,001 and up	3	5	3	15	14	40
Totals	149	46	30	22	19	263

The numbers of cells in the milk appeared to be correlated better with catalase concentration when the cell count was above 1 million per milliliter. Cells contain some catalase, but they probably account for only part of the catalase present in a sample of abnormal milk. In comparing the reproducibility of the catalase test results with the cell count determinations, the experimental technique employed should be considered. The cell counts in this study were performed in a much more accurate, yet more time-consuming manner than is possible in dairy plant or sanitarian's laboratory. Cell counts as usually performed in milk control work are quantitatively inaccurate, though of qualitative value.

The principal source of inaccuracy of the catalase test appeared to be in the measurement of the amount of oxygen. Bubbles of oxygen in the cream layer make an irregular surface for determining the height of the column of gas. This error in reading would be relatively less important with a greater concentration of catalase.

The catalase appeared to be fairly stable in these experiments during a holding period of six hours at room temperature. Some of the bacteria found in raw market milk can produce catalase, but there was no evidence that the catalase was produced or increased during the six-hour holding period at room temperature. In this respect, catalase tests are superior to resazurin or other reduction tests for mastitis. The bacteria in raw milk which is handled in the ordinary manner are present in insufficient numbers to affect the catalase test appreciably.[7] Nevertheless, the test should be made promptly after collection to avoid possible error.

Catalase is not present in significant quantities in normal milk except in early or late lactation. Therefore it represents one of the very few substances in milk exclusively associated with mastitis.

The CMT has an advantage of immediate results and it is very easy to perform. It is not a quantitative test and thus has obvious disadvantages in milk control work. In our opinion, it would be better to grade the results as negative, suspicious, and positive, rather than the five grades recommended. A serious disadvantage of the CMT is the

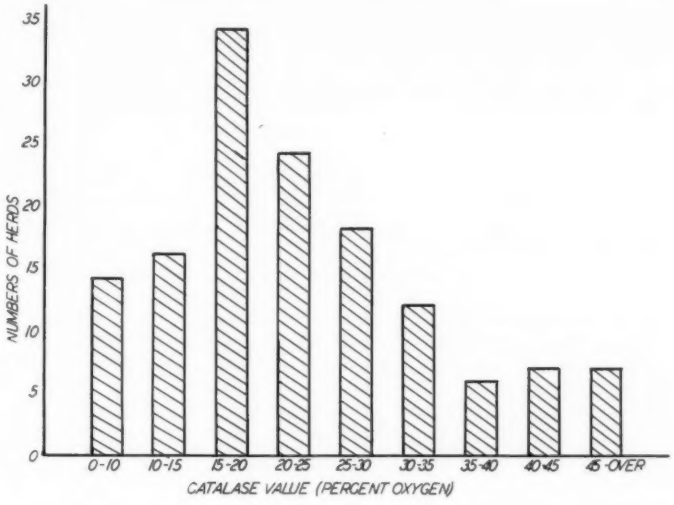

Fig. 3—The number of herds showing various degrees of catalase reaction as determined by a single test of milk taken from mixed milk of a day's production.

large number of doubtful (tr and "1") reactions. Nevertheless, the CMT is probably the most sensitive barn test available for mastitis. It would have value for use as an indication of the extent of a herd problem, or as a guide to palliative therapy. The CMT is not a reliable indicator of infected cows or of noninfected cows. Therefore it is definitely fallible as a guide to proper milking order or segregation.

Catalase and CMT reactive substances were found in milk from which cells had been removed by centrifugation. Both are certainly produced by body cells, as are all organic substances in milk. Inflammatory exudates derived from the breakdown of body cells from the udder and blood are the likely source both of catalase and the reactive substances in the CMT. Since the degree of breakdown of body cells varies with the cause, the duration, and the severity of mastitis, a variable relationship could be expected between the number of cells, the catalase, and the CMT-reacting substances, which are probably gel forming proteins such as fibrinogen.

The lack of correlation between bacteriologic tests and the test for inflammation is consistent with the evidence of other investigators and should serve as a warning for strong proponents of either kind of test. Tests for inflammation such as the CMT have little or no value as guides for quarantine, segregation, or sale because they do not indicate which animals are infected. Bacteriologic tests, however, have little value as indicators of the presence or severity of inflammatory changes, and are thus of doubtful value in the selection of cows for palliative therapy. Bacteriologic tests are too expensive for routine diagnosis in most states, but are certainly of value and their use is indicated in problem herds.

The lack of correlation between infection (as demonstrated by bacteriologic tests) and inflammation (as determined by catalase, cell count, or other means) suggests that other causes may be more significant than the infection detected. Further inves-

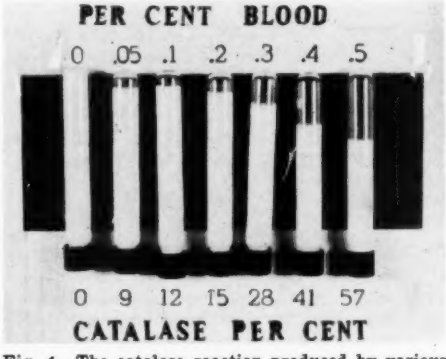

Fig. 4—The catalase reaction produced by various concentrations of whole blood in milk boiled to destroy all intrinsic catalase.

tigation is needed to establish the interactions of various causes that are producing the inflammatory reactions (mastitis). A search for new causes should be made in the substantial number of samples from inflamed quarters that show no recognized pathogens.

The catalase test should be applicable to quality control programs for both milk plants and sanitary inspectors. The test could be used routinely on herd milk samples as are the methylene blue and Brucella ring tests. From the results reported (fig. 3), it appears that herd milk with catalase values of about 40 per cent should be considered definitely abnormal, and values from 30 to 40 per cent should be considered suspect. These standards could vary from area to area. On the basis of the results obtained by us, the standards described would make about 10 per cent of the herds definitely abnormal and 13 per cent suspect for the presence of mastitis. The results of catalase tests for mastitis in herd milk perhaps could be utilized to stimulate the dairyman to keep abnormal milk out of the market supply and to call his local veterinarian for examination and treatment of individual cows.

SUMMARY AND CONCLUSIONS

A simple technique for determination of catalase in mixed herd milk was tested. This technique gave consistent results in replicate tests. The presence of catalase in milk was correlated with high body cell counts and the California mastitis test reaction, although the values were not uniformly parallel. The catalase test was compared with the California mastitis test, and the latter was found superior as a barn test on quarter samples where immediate results are required. For quality control tests on mixed herd milk, the catalase test is considered superior to the Breed count and the California mastitis test. No one test or combination of the three tests was a reliable indicator of the presence of bacterial infection in individual quarters.

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SUMMARIO IN INTERLINGUA

Le Test de Catalase, le Test Californian, e le Test de Numeration Cellular pro le Detection de Anormalitates in le Lacte

Un simple technica pro le determination de catalase in le lacte collective de un grege de vaccas esseva testate. Le technica produceva uniforme resultatos in essayos reduplate. Le presentia de catalase in le lacte esseva correlationate con alte numerationes de cellulas corporee e con le reaction in le test de California pro mastitis, sed le valores non esseva uniformemente parallel. Le test de catalase esseva comparate con le test de California pro mastitis, e il esseva trovate que iste ultime esseva superior como test de vaccheria con specimens ab quartieros in situationes ubi resultatos immediate es requirite. Pro tests del inspection qualitative del lacte collective de un grege, le test de catalase es considerate como superior al test de numeration cellular e al test californian. Nulle del tests individual e nulle combination de iste tests esseva fidel como indication del presentia de infection bacterial in quartieros individual.

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The Effect of Time and Temperature on the Gonadotrophic Potency of Pregnant Mare Serum

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RECENTLY, our evaluation of a pregnancy test for mares based on the presence of gonadotrophic hormones revealed almost 100 per cent accuracy.[13] Attaining this degree of reliability, however, requires not only experience in interpreting minor ovarian-uterine stimulation, but also knowledge of the expected hormonal concentration in serum at different stages of pregnancy, and how much hormonal activity a sample may lose from handling before arrival at the laboratory.

Many workers[1-7, 12] have studied the concentration of gonadotrophins in pregnant mare serum (PMS) at various stages of gestation; nevertheless, little information is available on the effect of storage and handling of pregnant mare serum. Often, in our diagnostic service, we must evaluate the effect of serum on the female reproductive organs of test animals on the basis of the date when the mare was bred as well as the time interval from collection of the sample to its arrival at the laboratory. A sample taken from a mare during the implantation period contains a concentration of gonadotrophins sufficiently high to give a positive reaction, even if some potency is lost; however, in samples taken during the other stages of pregnancy, any loss of potency may lead to error in a diagnostic test for pregnancy. Another important point to consider is that students of biology and research workers are often misled in their interpretation of the results obtained in dealing with gonadotrophic hormones because of a lack of knowledge of the degree of potency these substances may lose by repeated exposure to adverse temperature.

The following study was carried out to investigate the effect of time and temperature on the potency of gonadotrophic hormones present in pregnant mare serum, using a strain of mice sensitive to a single international unit (I.U.) of PMS.

From the New Jersey Agricultural Experiment Station, Rutgers, The State University, New Brunswick. Approved as Journal Series paper.

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MATERIALS AND METHODS

The experimental animals used in this investigation consisted of 536 immature female Swiss mice from a local commercial colony and from a colony of the same strain maintained in our laboratories. The mice were 22 days old and weighed 8 to 10 Gm. when treated. The experiment was carried out mainly during the months of May, June, October, and November.

The gonadotrophins were obtained by pooling PMS received in our laboratories for pregnancy diagnosis. The potency of this serum was determined in Sprague-Dawley female rats and Swiss female mice, and then standardized with commercial PMS.* The standardized serum was kept refrigerated at 4 C. Solutions for injection were made with sterile physiologic saline solution so that the desired concentration of gonadotrophins was contained in a single 0.5-ml. dose.

Animals were grouped before treatment with 1 I.U. of standardized PMS which had remained at room temperature for 0, 24, 48, or 72 hours. The same experimental design was used to study the effect of room temperature on PMS containing 2.0, 2.5, and 5.0 I.U. In all groups, each mouse was killed 48 hours after injection. Ovaries and uteri were dissected with the aid of a binocular loop and weighed in a Mettler balance (accurate to the fourth decimal point).

The material for histologic studies was fixed in Bouin's and Helly's fluid and 10 per cent formalin. Sections were cut at 5 and 8 μ and stained with hematoxylin and eosin and Masson's triple stain.

RESULTS

Controls.—A group of 24 mice given 0.5-ml. injections of physiologic saline solution were designated as controls. The mean ovarian weight for these mice was 2.69 mg., with a standard error of 0.19 (table 1; fig. 8), and the mean uterine weight was 9.98 ± 0.57 mg. (table 1; fig. 9).

The immature female mice in our colony were slightly more sensitive to gonado-

* Gonadin Serum, produced by Cutter Laboratories, Berkeley, Calif.

trophic hormones in spring than in other seasons of the year, and the ovaries of the control animals studied in May were slightly larger than those studied at other times of the year (table 1).

Mice Given 1 I.U. of Pregnant Mare Serum.—Twenty mice were each given 1 I.U. of PMS. Forty-eight hours later, 11 of the 20 had macroscopic signs of uterine or ovarian stimulation, or both, evidenced by enlargement of the uterus and oviduct, and some hemorrhagic spots on the surface of the ovary. The mean weight of the ovaries in this group was 2.70 ± 0.07 mg. (table 1; fig. 8). Histologically, these ovaries had relatively large crops of growing and secondary follicles with several layers of granulosa cells in various degrees of antrum formation (fig. 2). The mean weight of the uterus was 23.4 ± 3.08 mg., which is more than twice the mean weight of those in the control group (table 1; fig. 9).

The same solution of PMS used in the previous group was left for 24 hours at room temperature (25 C. average). A group of 20 mice, treated with this material at the same dosage administered before, developed noticeably less vascularized reproductive systems. The weight of the ovaries averaged 2.55 ± 0.34 mg. (table 1; fig. 8). The histologic changes in the ovaries in these mice were not much different from those in mice of the previous group. The crop of growing follicles appeared slightly reduced when compared with those in the mice given 1 I.U. of PMS. The average weight of the uterus was 17.62 ± 1.71 mg., which is significantly less than that of the previous mice.

The solution of PMS was kept for 48 hours at room temperature and then injected into 21 mice of another group. Macroscopic examination 48 hours after the injections showed evidence of gonadotrophic stimulation in 4 out of 21. The mean ovarian weight was 2.39 ± 0.06 mg. Histologically, the ovaries of these mice were not noticeably different from those of normal control mice of this age. The average weight of the uterus was 13.72 ± 1.76 mg. This mean weight of the uteri was less than that of the previous mice but still larger than that of the control group (table 1).

After 72 hours at room temperature, the same solution of PMS was injected into 53 mice. Examination of the ovaries and uteri of all the mice of this group showed no evidence of hormonal stimulation. The size and structure of the ovary remained in the infantile stage. The mean weight of the ovaries was 2.37 ± 0.04 mg. The mean uterine weight was 9.34 ± 0.29 mg. These results showed that the room temperature inactivated all detectable gonadotrophic hormones in 72 hours.

Mice Given 2 I.U. of Pregnant Mare Serum.—Following the same procedure used in the previous experiment with 1 I.U. of PMS, a similar experiment was undertaken using 2 I.U. of PMS. At necropsy 48 hours after the injection, ovaries and

TABLE 1—Summary of the Effect of Time and Room Temperature on the Potency of Pregnant Mare Serum (PMS) when Immature Female Swiss Mice Were Used for Detecting Hormonal Activity

PMS				
Dosage (I.U.)	Time held (hr.)	No. of animals	Ovarian weight (both) (mean ± s.e.) (mg.)	Uterine weight (mean ± s.e.) (mg.)
Control, NaCl 0.9%		24	2.69 ± 0.19	9.98 ± 0.57
1.0	0	20	2.70 ± 0.07	23.40 ± 3.08
1.0	24	20	2.55 ± 0.34	17.62 ± 1.71
1.0	48	21	2.39 ± 0.06	13.72 ± 1.76
1.0	72	53	2.37 ± 0.04	9.34 ± 0.29
2.0	0	33	3.96 ± 0.19	36.05 ± 2.16
2.0	24	22	3.32 ± 0.16	31.70 ± 2.95
2.0	48	21	2.58 ± 0.17	18.43 ± 1.65
2.0	72	39	2.53 ± 0.16	20.80 ± 1.63
2.5	0	43	4.47 ± 0.16	47.99 ± 2.25
2.5	24	34	3.18 ± 0.18	29.39 ± 1.96
2.5	48	21	3.08 ± 0.29	24.58 ± 3.04
2.5	72	35	2.28 ± 0.04	18.54 ± 1.78
5.0	0	41	7.08 ± 0.36	40.25 ± 1.78
5.0	24	28	5.13 ± 0.46	34.07 ± 1.83
5.0	48	36	3.61 ± 0.07	32.78 ± 2.19
5.0	72	45	2.33 ± 0.14	10.60 ± 0.67

S.E. = standard error of the mean.

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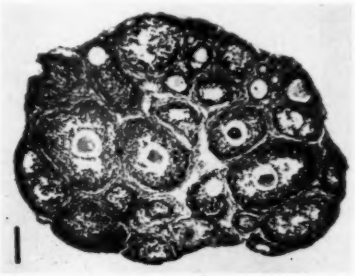


Fig. 1—Ovary of a 24-day-old control mouse killed in May. The size and histologic structure are better developed than those shown in figures 5 and 6. H & E stain; x 55.

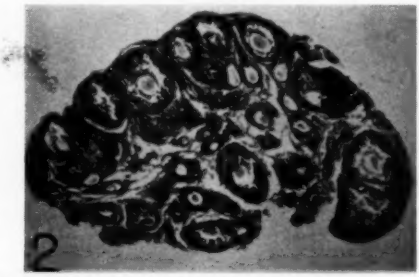


Fig. 2—Ovary of a 24-day-old mouse, given 1 I.U. of PMS and killed 48 hours later. H & E stain; x 55.

uteri of all these mice were found to have macroscopic evidence of gonadotrophic stimulation. The mean weight of the ovaries in these mice was 3.96 ± 0.19 mg. The histologic structure of these ovaries is shown (fig. 3). The mean weight of the uteri was 36.05 ± 2.16 mg., four times that of the normal controls (table 1; fig. 9).

The original solution containing 2 I.U. of PMS was used to test 22 mice after it had been kept at room temperature for 24 hours. At necropsy, evidence of gonadotrophic stimulation was still present in the ovaries and uteri of all the animals. Typical histologic structure of the ovaries is shown (fig. 4). The mean weight of the ovaries was 3.32 ± 0.16 mg. (table 1; fig. 8). The mean uterine weight was 31.70 ± 2.95 mg. Ovaries and uteri were slightly smaller than those of mice in the group tested with 2 I.U. of PMS.

The 48-hour solution was given to 21 mice. In 8 of 21, macroscopic signs of gonadotrophic stimulation of ovaries and uteri were observed. The histologic structure of the ovaries of these mice is shown (fig. 5). The mean ovarian weight was 2.58 ± 0.17 mg. The mean uterine weight was 18.43 ± 1.65 mg. The size of the ovaries remained like those of the control mice; however, the size of the uterus was twice that of the control uteri.

The 72-hour solution was tested in 39 mice. Examination of the ovaries at necropsy showed no evidence of hormonal stimulation; however, the mean weight of the uteri was still twice that of the control group. Histologically, the ovaries (fig. 6) were infantile. The mean weight of the ovaries was 2.53 ± 0.16 mg. The mean uterine weight was 20.80 ± 1.63 mg. (fig. 8, 9).

Mice Given 2.5 I.U. of Pregnant Mare Serum.—Necropsy of 43 mice given 2.5 I.U. of PMS showed macroscopic evidence of gonadotrophic stimulation. The mean

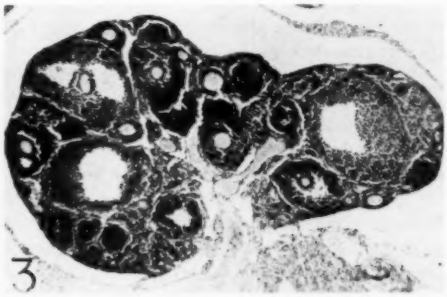
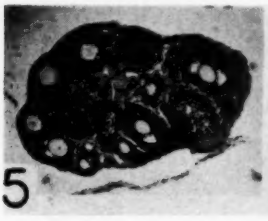


Fig. 3—Ovary of a mouse given 2 I.U. of PMS. The size and histologic structure show a fairly well-developed ovary. H. & E stain; x 49.

Fig. 4—Ovary of a mouse stimulated with 2 I.U. of PMS kept at room temperature (average 25 C.) for 24 hours. H & E stain; x 55.



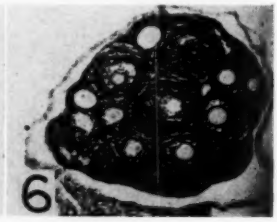


Fig. 5—Ovary of a mouse treated with 2 I.U. of PMS kept at room temperature 48 hours. H & E stain; x 55.

Fig. 6—Ovary of a mouse given 2 I.U. of PMS kept at room temperature for 72 hours. H & E stain; x 55.

weight of the ovaries was 4.47 ± 0.16 mg. Microscopically, the ovaries had growing follicles in various stages (fig. 7) and the majority of the ovaries had well-developed graafian follicles. Many of the uteri were highly distended and edematous. The average uterine weight was 47.99 ± 2.25 mg. (table 1; fig. 9).

The 24-hour solution was administered to 34 mice. The effect of gonadotrophic stimulation was observed macroscopically in all these mice. The mean weight of the ovaries was 3.18 ± 0.18 mg. In size and weight, these ovaries were significantly smaller than the ovaries of the mice in the previous group treated with 2.5 I.U. The histologic structure also reflected the loss of potency of this hormonal material. The uterine weight curve follows fairly closely the ovarian weight curve. The mean uterine weight was 29.39 ± 1.96 mg.

Twenty-one mice were given the 48-hour solution. The loss of potency of this solution was so evident that only 9 animals out of 21 had macroscopic evidence of ovario-uterine stimulation. However, the mean weight of the ovary was not much different from the preceding group (3.08 ± 0.29 mg.). The mean weight of the uteri was 24.58 ± 3.04 mg.

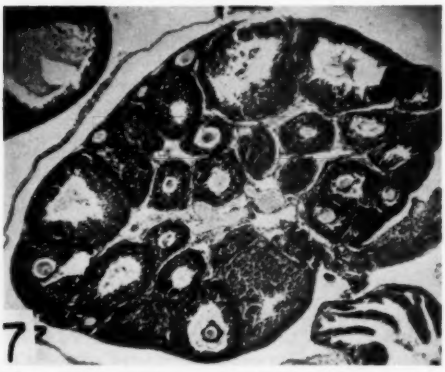


Fig. 7—Ovary of a mouse treated with 2.5 I.U. of PMS. H & E stain; x 49.

The same solution kept at room temperature for 72 hours was administered to 35 mice, only 25 per cent of which developed macroscopic signs of hormonal stimulation. Histologically, the ovaries resembled immature infantile ovaries. Their size was significantly smaller than those of mice treated with the 24- and 48-hour solutions. The mean weight was 2.28 ± 0.04 mg. The mean weight of the uteri was 18.54 ± 1.78 mg.

Mice Given 5 I.U. of Pregnant Mare Serum.—Forty-one mice were given 5 I.U. of PMS held for 0 hours at room temperature. Ovaries and uteri were greatly stimulated by this dosage. The mean ovarian weight in this group was 7.08 ± 0.36 mg. Histologically, these ovaries in many instances had large graafian follicles. The uterine weight was 40.25 ± 1.78 mg.

Twenty-eight mice were given the 24-hour solution. All mice had an intense gonadotrophic reaction. The ovarian weight, however, was significantly reduced. The mean weight of the ovaries was 5.13 ± 0.46 mg. Histologically, these ovaries were similar to those in the previous group, although the size and number of graafian follicles were not as large as those of the mice given PMS held at 0 hours room temperature. The mean weight of the uteri in this group was 34.07 ± 1.83 mg.

Thirty-six mice were given the 48-hour solution. The hormonal activity was so reduced that stimulated ovaries and uteri were found in only 23 out of the 36 mice. Histologically, these ovaries still had a considerable number of developing graafian follicles. The average weight of the ovaries was 3.61 ± 0.07 mg. The mean weight of the uteri was 32.78 ± 2.19 mg.

Forty-five mice were given the solution kept at room temperature for 72 hours. Only 10 out of 45 had macroscopic signs of

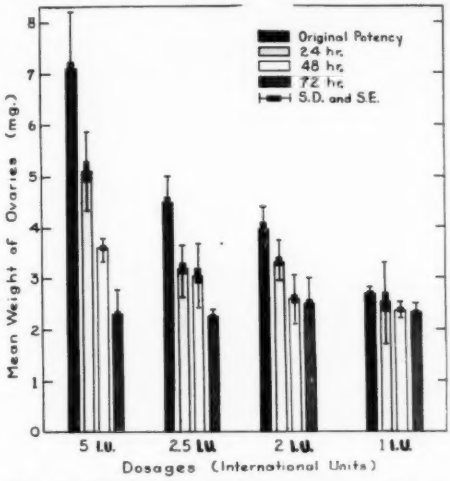


Fig. 8—Effect of room temperature on the potency of PMS, reflected on the ovarian weights.

gonadotrophic stimulation. Histologically, these ovaries were similar to those of the control mice. The mean ovarian weight was 2.33 ± 0.14 mg. (table 1; fig. 8). The mean weight of the uteri was 10.60 ± 0.67 mg. These results showed that the PMS solution after 72 hours at room temperature had completely lost its potency.

DISCUSSION

Reports in the literature indicate that the gonadotrophic hormones, being of protein nature, are quite sensitive to high temperatures. Fevold [10] reported that boiling for an hour inactivated FSH as well as LH. He also showed that FSH keeps well in distilled water at a temperature of 4 C. The data presented in the present report demonstrate that equine gonadotrophins, at the levels used, lost practically all potency gradually during the observed period of 24 to 72 hours, when room temperature was within a small range of variation. However, when the temperature changed to low or high during the 24-hour period, the variation was reflected in the hormonal activity of the solutions; that is, the hormonal activity of the solution was inversely proportional to the room temperature. It may be argued that this experiment would be better designed if the solutions used were kept at a constant temperature instead of room temperature; however, in designing the experiment we intended to study the effect of ambiental temperatures under the conditions in which the specimens for diagnosis and the hormones for experimental work are handled.

Table 1 shows the average weight of ovaries of the control mice. These ovaries were slightly larger than those of mice given the 72-hour solution, the ovaries of which resembled in size and structure those of the control mice killed with the experimental animals during the fall and winter. We include here the control group studied in May to show the seasonal variations in ovarian weights in the strain of mice used in this experiment. Figure 1 shows the ovary of a 24-day-old mouse killed in May. The seasonal variation is still more evident

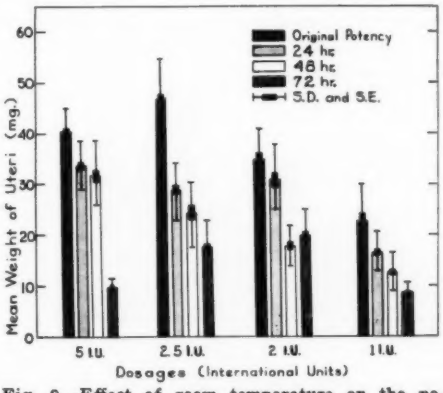


Fig. 9—Effect of room temperature on the potency of PMS, measured in terms of uterine weights.

in the histologic structure of the ovaries than in ovarian weights (compare with fig. 5 and 6). It is also observed that the mice of our colony are more sensitive to gonadotrophic hormones in the spring than in fall and winter. This seasonal variation plays an important part in a year-around experiment. By always giving injections at the same hour of the day we avoided the interference of diurnal variation in response to gonadotrophic hormones, an important phenomenon pointed out by Everett and Sawyer [8,9] and more recently by Lamond and Braden.[11]

SUMMARY

This study was designed to show the effect of room temperature on the potency

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of pregnant mare serum (PMS) within a 72-hour period. The experimental animals used were female Swiss Albino mice, 22 days old at the time of injection and killed 48 hours later. The PMS was obtained from blood serum sent to our laboratories for diagnosis of pregnancy in mares and was assayed for potency in international units (I.U.).

The experiment, involving 536 mice, was carried out as follows: mice in one group were treated with 1 I.U. of PMS held 0 hours at room temperature; mice in another group were given the same solution after 24 hours at room temperature; those in a third group were given the same solution of PMS held 48 hours at room temperature; those in a fourth group were given the same solution after it had remained 72 hours at room temperature. The same experimental design was used to study the effect of room temperature on 2.0, 2.5, and 5.0 I.U. doses of PMS.

The results showed that room temperature inactivates practically all potency of gonadotrophins at the levels used in this experiment during a 72-hour period. The criteria used for testing gonadotrophic activity were: macroscopic appearance of the reproductive organs, ovarian and uterine size and weights, and histologic structure of the ovaries. The curve of the ovarian weights followed closely the curve of the uterine weights.

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SUMMARIO IN INTERLINGUA

Le Effecto de Tempore e Temperatura Super le Potentia Gonadotrophic del Sero de Cavallas Pregnante

Le presente studio esseva planate pro monstrar le effecto del temperatura normal de interior super le potentia gonadotrophic del sero de cavallas pregnante (SCP) intra le limites de un periodo de 72 horas. Le animales experimental usate esseva muses albin switze. Al tempore del injection illos habeva 22 dies de etate. Illos esseva sacrificate 48 horas plus tarde. Le SCP esseva obtenite ab le specimens de sero sanguinee que es inviate a nostre laboratorios pro le diagnose de pregnantia in cavallas. Le potentia constatate esseva exprimite in unitates international (U. I.).

Le experimento utilisava 536 muses. Le methodo experimental esseva le sequente: Un gruppo de muses esseva tractate con 1 U. I. de SCP post 0 horas de immagasinage al temperatura normal de interior; un secunde gruppo recipeva le mesme tractamento con SCP immagasinate al temperatura normal de interior durante 24 horas; in un tertie gruppo le immagasinage esseva de 48 horas; in un quarte, de 72 horas. Le mesme schema esseva usate pro doses de 2, 2,5, e 5 U. I. de SCP.

Le resultatos monstrava que le temperatura normal de interior effectua un inactivation quasi complete del potentia gonadotrophic del dosages usate, intra un periodo de 72 horas. Le criterios usate pro le determination del activitate gonadotrophic esseva (1) le apparentia macroscopic del organos de reproduction, (2) le dimensiones e pesos ovarian e uterin, e (3) le structura histologic del ovarios. Le curva del pesos ovarian sequeva vicinmente le curva del pesos uterin.

Propagation of Hog Cholera Virus in Tissue Culture and Its Application to Vaccine
I. Propagation of the Virus in Tissue Culture

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SINCE Hecke [9] was successful, in 1932, in propagating hog cholera virus (HCV) in cultures of swine choroid plexus, bone marrow, lymph node, and spleen, various methods of cultivating the virus have been described.[1-5, 10, 11] There is now no doubt that the virus propagates in cultures of different pig tissues and can be passaged serially in them. The application of a tissue-culture method to the production of an inactivated vaccine, however, has not yet been reached in practice.

The objective of this study was to establish a method suitable for the mass production of highly virulent culture which might be useful as the source of an inactivated vaccine.

Before starting the study, the following two steps were taken. First, a type of culture was adopted in which adult pig tissues, minced into fragments, were cultivated. This type of culture was selected because it was considered to be the most economical and advantageous in practice for the mass production of the virus. Second, pilot experiments with Newcastle disease virus (NDV) were conducted for every detail of the culture technique. Such pilot experiments could be performed with relatively small expenditure. It was assumed that they might give analogies and contribute to minimize the scale of the main experiments on HCV which are always expensive and require pigs as experimental animals.

Pilot experiments had to be conducted under culture conditions which were similar, if possible, to those expected in the HCV experiments. For this, NDV was cultivated in tissue fragments of adult chicken spleen. The results of these experiments have been previously reported.[6-8] Results of the HCV experiments were surprisingly coincidental, in principle, with those of the pilot experiments. Most of the factors which had served for the improvement of the titer of NDV were found effective in raising the titer of HCV and, under the best culture condition available, the whole culture gave a titer of $10^{-6.7}$ to $10^{-7.5}$ for NDV or a titer level of 10^{-8} of HCV. This report describes the advance in the culture method for the propagation of HCV.

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MATERIALS AND METHODS

Pigs.—Young Yorkshire pigs, raised in the area of Nagano prefecture and having no history of hog cholera infection or vaccination, were collected for the source of tissue and serum to be used in the culture as well as for experimental purposes. They weighed 30 to 50 kg. In this report, they will be referred to as adult pigs. In one experiment, 85-day-old pig embryos were harvested from a pregnant sow which had been slaughtered.

Tissues.—In the preliminary experiment, spleens, lungs, livers, lymph nodes, and red bone marrow of embryonic and adult pigs were used comparatively for cultivation. In the main study, however, spleens of adult pigs were used exclusively.

Virus.—The seed virus used was virulent serum obtained from pigs inoculated with the ALD strain of hog cholera virus. It was stored at −40 C. or below until use.

Culture Medium.—The culture medium used was 10 per cent untreated pig serum in Tyrode's solution to which 100 units of penicillin and 1 mg. of streptomycin per milliliter had been added. Its starting pH was between 7.4 and 7.8. The culture medium was usually inoculated with the virulent serum in an amount of 10^{-2} to 10^{-3} of its volume and then distributed into culture vessels.

Tissue Fragments.—Tissues removed from exsanguinated pigs were minced in Tyrode's solution, with scissors, into fragments approximately 1 cmm. in size and washed by changing Tyrode's solution several times.

Tissue-Fixing Material.—Fragments of tissue were fixed on Japanese rice paper. The paper was cut to pieces of the required size, washed with distilled water, dried, and sterilized by steam under high pressure.

Vessels.—The vessels used were four different sizes of ordinary vaccine bottles, the capacity and diameter of which were 20 ml. and 32 mm., 50 ml. and 40 mm., 100 ml. and 50 mm., and 500 ml. and 75 mm., respectively, and a test tube 18 mm. in diameter and 185 mm. in length. Unless otherwise stated, each bottle contained the culture medium to one tenth of its capacity, and the test tube, in an amount of 2 ml. All the vessels were cotton-plugged.

Method of Tissue Culture.—The techniques for the tissue culture have been improved several

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TABLE 1—Infectivity of Hog Cholera Virus in Cultures Containing Embryonic and Adult Tissues

Experiment (No.)	Culture: Tissue: Source	Tissue: Concentration (%)	Culture: Culture method	Culture: Days of cultivation	Days of incubation in pigs inoculated with various dilutions: 10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
4	Embryonic spleen	1	Suspension	3	....	3.5	3.0	7.0	2.5	Neg.
	Embryonic lung	1	Suspension	3	....	3.0	4.0	Neg.	2.5	....
	Embryonic liver	1	Suspension	3	....	....	Neg.	....	....	....
	Embryonic lymph node	1	Suspension	3	....	....	Neg.	Neg.	....	....
	Adult spleen	1	Suspension	3	....	2.0	2.5	Neg.	Neg.	....
	Adult lung	1	Suspension	3	....	....	Neg.	Neg.	....	....
	No tissue	..	.....	3	Neg.	Neg.	....	....	....	....
5	Adult bone marrow	1	Fixing	3	....	....	Neg.	Neg.	....	....
6	Adult bone marrow	1	Fixing	3	....	....	Neg.	Neg.	....	....

Neg. = Negative (not infected).

times. In principle, however, the culture methods employed may be classified as follows:

Suspension Method.—Small culture bottles (20-, 50-, and 100-ml. capacity) were used and 1 per cent tissue fragments were suspended in the culture medium in each bottle. The cultures were kept in an incubator at 37 C. for three days; they were agitated slightly every morning and evening.

Tissue-Fixing Method.—Only 20-ml. bottles were used. Tissue fragments varying from 3 to 7 per cent in amount were fished by a needle, one by one, and disseminated on a round piece of tissue-fixing paper, the diameter of which was a little less than that of the bottle. The tissue fragments were fixed on the paper instantly. Then the paper piece was slightly rolled and forced into the bottle through the mouth. The paper was then extended with its back toward the bottom of the bottle, and the culture medium was poured on it. Cultivation was made as mentioned in the preceding method. The paper with the tissue fragments maintained its half-floating state during the culture period.

Rolling Method.—In this method of culture, a test tube or a 500-ml. bottle which had a piece of tissue-fixing paper fixed on the inside wall was used. The paper for the test tube was a rectangle approximately 2 by 5 cm. Before use, it was inserted into the tube and fixed to the wall moistened by a minute amount of Tyrode's solution dropped into the tube. The paper for the 500-ml. bottle was a rectangle approximately 12 by 23 cm. The bottle, with the paper inside, was sterilized by steam, so that the paper was fixed to the wall by moisture.

When the culture vessel was ready, 3 per cent tissue fragments (*i.e.*, approximately 0.06 Gm. for the test tube and 1.5 Gm. for the 500-ml. bottle, suspended in a small amount of Tyrode's solution) were placed on the bottom by means of a pipette with a relatively wide tip, and then spread over the paper by turning the vessel on its side and rolling it slowly. The vessel was then held upright for a few minutes so that Tyrode's solution might move to the bottom and the tissue fragments might be fixed steadily on the paper. After discarding Tyrode's solution, the culture medium (2 ml. for the test tube or 50 ml. for the 500-ml. bottle) was pipetted in, and the vessel was put on the rolling drum which was fitted in an incubator at 37 C. and rolled eight times an hour. The period of culture varied from two to four days.

Medium-Changing Method.—A culture in a 500-ml. bottle was prepared as described for the rolling method, except for two modifications: The tissue fragments were cultivated at higher concentration (10% instead of 3), and the culture medium was renewed twice daily for the first two days of the three days' cultivation.

Titration of Virus.—The entire contents of two or three culture vessels were pooled and emulsified in a Waring blendor or with mortar and pestle. The emulsion thus prepared was subjected to virus titration immediately or after preservation in a refrigerator at −40 C. for some days. The emulsion, either fresh or thawed, was centrifuged at 2,000 r.p.m for ten minutes, and the supernatant fluid was serially diluted by a factor of 10 with buffered NaCl solution of pH 7.4. Often, however, only one or two dilutions presumed to be close to the virus titer were used for inoculation in order to economize on test pigs. Prior to inoculation, all samples were further diluted five times so as to inoculate 1 or 2 pigs with a subcutaneous dose of 5 ml.

Hog cholera infection was diagnosed on the basis of characteristic clinical and postmortem

TABLE 2—Infectivity of Hog Cholera Virus in Cultures Containing Adult Spleen—Tissue-Fixing Method

Experiment (No.)	Culture: Concentration of tissue (%)	Culture: Days of cultivation	Days of incubation in pigs inoculated with various dilutions: 10^{-5}	10^{-6}	10^{-7}
7	3	3	4.0	4.0	Neg.
8	5	3	4.0	3.0	3.0
9	3	3	....	6.0	Neg.
	5	3	....	3.0	Neg.
	7	3	....	Neg.	Neg.
11	5	3	....	3.5	Neg.
16	3	3	....	3.5	Neg.

findings. The incubation period was defined as the interval of days between inoculation and the onset of fever (40.1 C. or higher) or the appearance of other clinical signs (inactivity and loss of appetite). The virus titer was expressed by the highest infective dilution; the additional dilution of 1:5, made prior to inoculation, was excluded since the final dilution was used in a dose of 5 ml.

All cultures were simultaneously tested for bacterial contamination.

RESULTS AND DISCUSSION

Preliminary Experiments.—In three preliminary experiments (table 1), propagation of the virus was compared in cultures of four embryonic tissues (spleen, lung, liver, and lymph node) and of three adult tissues (spleen, lung, and red bone marrow). The culture technique applied was the most simple suspension method for all the tissues, except for adult red bone marrow which was cultivated by the tissue-fixing method.

After completion of cultivation for three days, the cultures made with embryonic spleen and embryonic lung were both infective up to a dilution of 10^{-7}, and the culture made with adult spleen, infective up to a dilution of 10^{-5}. All the other cultures were found avirulent at the lowest dilution tested (10^{-5}), and the control culture which contained no tissue became avirulent at 10^{-3}.

Compared with the results of other cultures, as well as those of the control, the occurrence of virus was of little doubt in the cultures of embryonic spleen and lung and was highly probable in the culture of adult spleen. Taking into consideration the circumstances mentioned in the introduction of this report, it was decided to adopt adult spleen for tissue culture in further investigation.

Tissue-Fixing Method.—In the pilot study, tissue fixing with adult chicken spleen usually gave a NDV titer one factor higher than the suspension method with the same tissue. To determine effect of the former method on culture with adult pig spleen for propagation of HCV, five concurrent experiments (table 2) were carried out in which the effect of tissue concentration within a range of 3 to 7 per cent was studied.

Of the cultures tested, one which had been made with 7 per cent tissue was found noninfective at 10^{-6}. All the others which had been made with 3 or 5 per cent tissue were proved infective at this dilution; one of them was even infective at 10^{-7} (table 2). Comparison of these virus titers with those obtained in the preliminary experiments gives an impression that the tissue-fixing method provided better conditions than the suspension method for the propagation of HCV as well as of NDV.

TABLE 3—Infectivity of Hog Cholera Virus in Cultures Containing Adult Spleen—Rolling Method

Experiment (No.)	Culture				Days of incubation in pigs inoculated with a 10^{-7} dilution	
	Vessel	Concentration of tissue (%)	Amount of medium (ml.)	Days of cultivation		
19	Test tube	3	2	3	5.5	5.5
21	Test tube	3	2	3	Neg.	Neg.
23	Test tube	3	2	3	3.5	Neg.
25	500-ml. bottle	3	50	3	3.5	4.0
27	500-ml. bottle	3	50	2	Neg.	4.0
	500-ml. bottle	3	50	3	Neg.	Neg.
	500-ml. bottle	3	50	4	Neg.	Neg.
29	500-ml. bottle	3	50	2	Neg.	Neg.
	500-ml. bottle	3	50	3	7.0	Neg.

Rolling Method.—The periodic exposure of the tissue to air by rolling was shown to improve the titer of NDV in culture of adult chicken spleen. A similar rolling method was studied for effect on the propagation of HCV in culture of adult pig spleen, making three experiments with test tubes and three with 500-ml. bottles (table 3). In two of the experiments using 500-ml. bottles, cultures of two or four days were prepared simultaneously with regular cultures of three days.

Two pigs were inoculated with only one dilution (10^{-7}) of the culture. Although

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TABLE 4—Infectivity of Hog Cholera Virus in Cultures Containing Adult Spleen—Medium-Changing Method

Experiment (No.)	Culture			Days of incubation in pigs inoculated with various dilutions			
	Concentration of tissue (%)	Amount of medium (ml.)	Days of cultivation	10^{-7}	10^{-8}		10^{-9}
33	10	50	3.0	....	2.5	2.5	Neg.
34	10	50	3.0	....	Neg.	Neg.	....
35	10	50	3.0	....	Neg.	Neg.	....
36	10	50	2.0	2.0	2.0	Neg.	....
	10	50	2.5	2.5	Neg.	....	....
	10	50	3.0	Neg.	Neg.	....	....
43	10	50	2.0	....	Neg.	....	....
	10	50	3.0	....	2.5	....	....
45	10	20	3.0	....	3.5	....	....
	10	100	3.0	....	3.5	4.0	....
46	10	50	3.0	....	2.0	....	....

no precise virus titers were obtained by such tests, the results from the regular cultures indicated that their mean titer might be near the dilution inoculated, since, of the three cultures, either in test tubes or in 500-ml. bottles, one was infective to both pigs, a second infective to 1 pig, and the remaining one not infective to either pig. When compared with the results obtained from the preceding method, these results were also indicative that the rolling had a favorable effect (table 3).

The culture of two days gave one better and one worse result, and the culture of four days gave one worse result than the regular culture. Therefore, a change in the time of culture was not suggested.

Medium-Changing Method.—It was found, in the pilot experiments, that the use of tissue fragments in relatively high concentration and frequent changes of culture medium during the earlier stage of cultivation favorably influenced the propagation of NDV in culture by the rolling method. Seven experiments were performed to study a similar medium-changing method for the propagation of HCV in cultures in 500-ml. bottles (table 4). The time of culture was regularly three days in all experiments except two (No. 36 and 43) where cultures of two and two and one-half days were additionally tried. The volume of culture was regularly 50 ml. in all experiments, except one (No. 45) in which it was 20 and 100 ml., in order to compare results.

Results were determined by inoculating 1 or 2 pigs with one dilution (10^{-8}) of the culture. The effect of culture time was again indistinct within the range of two to three days. The two cultures in which the volume was modified to 20 and 100 ml., respectively, were not significantly different from the regular culture containing 50 ml., since both cultures were found infective. Of 11 cultures considered, regardless of such small modifications in techniques, six proved infective.

It is obvious that conditions for propagation of HCV in cultures of adult pig spleen were again improved by application of the medium-changing method. The mean virus titer of the culture thus obtained appeared to be near 10^{-8}.

The incubation period in an infected pig is helpful, to a certain extent, in judging the strength of the virus inoculated. Incubation periods in 8 pigs infected with 10^{-8} dilution of cultures by the medium-changing method were between 2.0 and 4.0 days (av. 2.9 days), while those in 7 pigs infected with 10^{-7} dilution of cultures by the rolling method were between 3.5 and

TABLE 5—Comparison of Effects of Culture Methods on Propagation of Newcastle Disease Virus (NDV) and Hog Cholera Virus (HCV)

Tissue* cultivated	Culture method	Material titrated	Titer (−log) of	
			NDV	HCV
Adult spleen	Suspension	Whole culture	4.8–5.5	5
	Tissue-fixing	Whole culture	5.7–6.5	6
	Rolling	Whole culture	6.0–6.7	7
	Medium-changing	Whole culture	6.7–7.5	8
		Tissue	8.5	....
Embryonic spleen	Suspension	Whole culture	6.7–7.5	7

* Tissues were harvested from chicks or chick embryos for NDV and from pigs or pig embryos for HCV.

7.0 days (av. 4.7 days). The distinct reduction in the incubation period suggested that the elevation of virus titer following the improvement of the culture method, from the rolling to the medium-changing, may have been by more than one dilution.

Serial Passage of HCV in Tissue Cultures.—Serial passage of HCV in cultures of adult pig spleen was carried to the eighth generation. The technique employed was the tissue-fixing method up to the seventh passage, and the rolling method for the last passage. The initial virus used was the virulent serum which was inoculated into the first culture with the final dilution of 10^{-3}. Further cultures were inoculated with the fluid of the preceding culture in one-tenth volume of the culture fluid.

Virus titers of the fifth and eighth cultures measured in pigs were both 10^{-6}. In calculation, the infective dilution of the fifth culture is equivalent to 10^{-13} of the initial virus and that of the eighth culture, to 10^{-16}. At these two passages, control cultures were simultaneously prepared, to which a fresh virus was inoculated instead of the passage virus. Virus titers of the control cultures were 10^{-6} and 10^{-7}, respectively. No significant difference was found between the passaged and fresh viruses in ability to propagate in tissue culture.

Most of the culture conditions which had served for improving the propagation of NDV in tissue cultures of adult chick spleen were found effective in raising the titer of HCV in tissue cultures of adult pig spleen. The titers of both viruses obtained by the four different culture methods were compared and tabulated (table 5).

When chicken spleen was cultivated under optimal conditions, the propagation of NDV was better in the cultivated tissue ($l.d._{50}$: $10^{-8.5}$) than in the spleen of artificially infected birds ($l.d._{50}$: $10^{-7.0}$). Coincidentally, HCV appeared to propagate more vigorously in cultivated pig spleen than in the same tissue of artificially infected animals. The average virus titer (10^{-8}) of the whole culture which was prepared by the medium-changing method and which consisted of 10 per cent tissue substance and 90 per cent fluid, was on the same level as that of the solid tissue of spleen obtained from artificially infected pigs.

Summary

Guided by the results of pilot experiments with Newcastle disease virus, various culture conditions were used in the propagation of hog cholera virus in cultures of adult pig spleen tissue fragments.

The most simple method of culture, in which the tissue fragments were suspended in Tyrode's solution plus 10 per cent normal pig serum, gave a final virus titer of 10^{-5}. Main improvements in culture conditions which were effective in raising the final virus titer of the culture were:

1) Tissue fragments were fixed and cultivated on a rolling drum.

2) A relatively high concentration of tissue fragments was cultivated under adequate ventilation and frequent changes of culture medium. For this purpose, the tissue concentration was made to 10 per cent, air was allowed to pass freely through a cotton plug placed at the neck of the culture vessel, and the culture medium was renewed twice daily for the first two days of the three days' cultivation.

The virus titer of a whole culture which had been prepared under the improved culture conditions was roughly 10^{-8}. This titer was comparable to that obtained from the solid spleen tissue of artificially infected pigs.

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SUMMARIO IN INTERLINGUA

Le Propagation de Virus de Cholera Porcin in Histoculturas e Su Application a Un Vaccino

I. Propagation del Virus in le Histocultura

Post experimentos orientatori con virus de morbo de Newcastle (que es relativemente incostose), varie schemas de culturation esseva usate in le propagation de virus de cholera porcin in culturas de fragmentos de tissu del splen de porcos adulte.

Le methodo le plus simple, in que le fragmentos de tissu essva suspendite in solution de Tyrode continente 10 pro cento de normal sero porcin, produceva un titro final de virus de 10^{-5}. Le principal meliorationes del schema de culturation, resultante in le efficace augmento del titro final de virus, esseva le sequentes:

1. Le fragmentos de tissue esseva fixate e culturate intra receptaculos rotante.

2. Un relativemente alte concentration de fragmentos de tissu esseva culturate sub conditiones de ventilation adequate e con le renovation frequente del medio de culturation. Pro iste objectivo, le concentration de tissu esseva augmentate a 10 pro cento. Un libere provision de aere esseva assecurate per un tampon de coton in le collo del flasco de culturation, e le medio esseva renovate duo vices per die durante le duo prime dies del periodo de tres dies de culturation.

Le titro de virus de un cultura total, preparate sub le meliorate conditiones, esseva circa 10^{-6}. Iste titro esseva comparabile a illo obtenite ab le tissu solide del splen de artificialmente inficite porcos.

Cytochemical Reactions of Porcine Spermatids and Spermatozoa

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THE DETAILED description of boar spermatozoa recorded by Retzius,[22] based on observations through the light microscope, has been substantiated and augmented by modern techniques of study. Kessler [14] has shown, by means of the electron microscope, that the axial filament is composed of 11 fibrils. He reported that two of the fibrils were located in the center, and the remaining nine were arranged in a circle around them. Herrnleben [13] has recorded detailed measurements of spermatozoa of all the domestic animals, including the boar. Hancock,[12] after using both the phase contrast microscope and electron microscope, clarified some of the structural features and terminology relating to boar spermatozoa. However, aside from the periodic acid-Schiff technique used by Hancock, little use has been made of cytochemical methods in the study of boar spermatozoa.

Cytochemical methods have been used to study the spermatozoa of other species. Wislocki [25] used acid and alkaline phosphatase, Sudan black B, Bodian's protargol, and other cytochemical techniques in the study of human spermatozoa and seminal plasma.

Friedlander and Fraser [8] used techniques for polysaccharides, nuclear material, lipids, cholesterol, and phosphatase enzymes in the study of ram spermatozoa. Melampy *et al.*[19] employed similar techniques in the study of bull spermatozoa. In the studies of species other than swine, little attention has been paid to the age of the sperm cells or the histochemical changes in the spermatid during spermiogenesis.

This study was undertaken to determine the histochemical and cytochemical reactions of boar spermatozoa from different areas of the male reproductive tract and also after ejaculation and storage. The possibility of finding various reactions due to metabolic changes after storage and aging of the spermatozoa motivated this study. If techniques to demonstrate these changes could be found, they would be valuable tools in predicting the storage and fertilizing capabilities of various semen samples for artificial insemination.

MATERIALS AND METHODS

Samples of semen from 5 Yorkshire boars, 16 months old, were collected by means of an artificial vagina and dummy sow as described by Lovell.[5] The boars had been used for breeding experiments earlier and had been found to be fertile. Motile spermatozoa were present in all samples. Both fresh and aged semen samples were studied by means of the phase microscope to observe the morphology of unstained living and dead cells. Smears were made from fresh semen immediately after collection and also from semen which had been stored for varying periods of time, up to 120 hours. Control smears were made of spermatozoa which had been washed by repeated centrifugation, using Ringer-phosphate solution buffered at pH 7.2. Smears of epididymal fluid were also made, after castration, to study the morphology and staining characteristics of spermatozoa from the epididymis. Tissue blocks were collected from the testicle, epididymis, and ductus deferens in order to study the development and maturation of spermatozoa. Three sites were sampled as illustrated (fig. 1). The tissue blocks were fixed in the appropriate fluid for the stain to be used, embedded in paraffin, and sectioned at 6 to 8 μ. The sections were mounted on glass slides with Mayer's albumin, deparaffinated, and stained by the same methods used for smears. All smears were air-dried for three to four hours prior to fixation and staining. The following fixatives, staining procedudes, and cytochemical reactions were used.

Periodic Acid-Schiff Procedure.—Smears and tissue blocks were fixed for 24 hours in Zenker's fixative. Staining was done according to McManus.[18] In order to remove polysaccharides such as glycogen, control smears and sections were incubated in diastase one hour at 37 C. before treatment.

Acid Phosphatase.—Smears and tissue blocks were fixed in chilled acetone for 24 hours. Smears and sections were incubated in sodium glycero-

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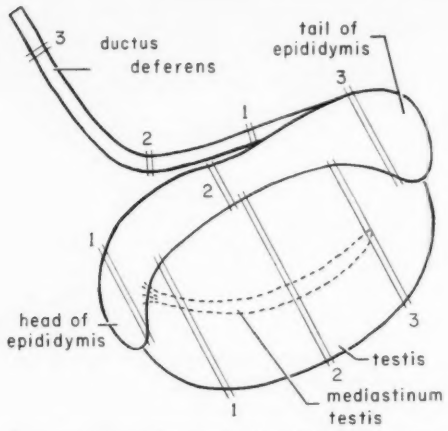

Fig. 1—Schematic drawing of genital tract of boar. Arabic numerals indicate locations from which tissue blocks were taken for sectioning.

phosphate substrate at pH 5.0 for 48 and 72 hours at 37 C. as outlined by Gomori.[9]

Alkaline Phosphatase.—Smears and tissue blocks were fixed in 80 per cent ethyl alcohol at 4 C. for 12 to 24 hours. This reaction was carried out at pH 9.4 for four, eight, and 24 hours according to Gomori.[9] Sodium glycerophosphate was used as a substrate.

Sudan Black B.—Smears and tissue blocks were fixed in 10 per cent formalin for 6 and 24 hours. Slide preparations were stained in a saturated solution of Sudan black B in 70 per cent alcohol for times varying from one to two hours. After staining, they were dipped into 70 per cent ethyl alcohol, washed in distilled water, and mounted in Farrant's solution.

Gomori's Reticulum Silver Stain.—The smears and tissue blocks were fixed for 24 hours in 10 per cent formalin. Staining was carried out according to Gomori's reticulum method.[17]

Trichrome Stain.—Following fixation for 24 hours in Zenker-formol solution, the smears were stained by means of the Crossman[5] modification of Mallory's connective tissue technique.

Feulgen Test.—Smears and tissue blocks were fixed 12 to 24 hours in 10 per cent formalin and stained by means of the Feulgen test of Stefano[23] for desoxyribonucleic acid (DNA).

Methyl Green and Pyronine.—For the differentiation of ribonucleic acid, the methyl green and pyronine mixture of Unna and Pappenheim[24] was used after 24 hours fixation in a solution of 5 ml. of glacial acetic acid, 5 ml. of 40 per cent formaldehyde solution, and 90 ml. of 80 per cent ethyl alcohol. Control sections and smears were incubated in a 0.01 per cent solution of ribonuclease at 60 C. for one hour.

Casarrett Morphologic Stain.—Smears were fixed in 50 per cent ethyl alcohol and 50 per cent ether solution and were stained according to the Casarrett[3] technique.

Baker's Acid Hematin Test for Phospholipid.—This procedure and the associated control, or pyridine extraction test, were carried out as prescribed by Baker.[1] The smears for hematin treatment were fixed in formol calcium and the control smears in Baker's "weak Bouin's fluid."

Aceto-Carmine Stain. Smears were fixed and stained simultaneously in aceto-carmine solution as recommended by Green.[10]

Gram's Stain.—Hucker's modification of Gram's method, as described by Guyer,[11] was used.

Hematoxylin and Eosin.—Sections and smears were stained with Harris' alum hematoxylin[17] and the precipitated eosin Y technique.[20] This technique was used after fixation for 24 hours in 10 per cent neutral formalin.

Bodian's Protargol Method.—Smears and tissue blocks were fixed in Bodian's fixative No. 2 (80% ethyl alcohol, 90 ml.; 40% formaldehyde solution, 5 ml.; and glacial acetic acid, 5 ml.). Staining was carried out according to Bodian's method as modified by Dawson and Barnett.[6]

Glycogen.— Smears and tissue blocks were fixed for 24 hours in chilled Rossman's fluid (90 ml. of absolute alcohol saturated with picric acid and 10 ml. of 40% formaldehyde solution). Staining was carried out by means of the Bauer-Feulgen method.[2] Control smears and sections were treated with saliva or diastase at 37 C. for one hour before staining.

OBSERVATIONS

Periodic Acid-Schiff Reaction.—All stages of spermiogenesis can be observed in sections of boar testicle treated with this technique. According to the classification of Leblond and Clermont,[16] based on studies of rat testes, the sequence of changes can be divided into four phases: Golgi, cap, acrosome, and maturation. These phases, as they occur in the boar testes, are shown (fig. 2a–17b, 38–42).

The Golgi phase (fig. 2a–4, 38) begins with the spermatids immediately after the division of the secondary spermatocytes. These early spermatids are circular cells with centrally located nuclei (fig. 2a). The acrosomic granules first appear as a few bright-red granules at one side of the nucleus (fig. 2b). These granules coalesce into one large granule (fig. 3). The acrosomic vesicle develops around this granule, marking the end of the Golgi phase (fig. 4, 39).

The cap phase (fig. 5–8) develops after

the acrosomic vesicle collapses (fig. 5). A membrane spreads out from the granule over the surface of the nucleus (fig. 6–8) until the cap covers two thirds of the nucleus (fig. 9).

The acrosome phase is characterized by the thinning of the cytoplasm on one side of the nucleus and the development of the sperm tail (fig. 9, 10, 41). The cytoplasm aggregates around the base of the tail, the nucleus becomes elongated, and the manchette becomes evident (fig. 11). Further development of the manchette and elongation of the nucleus produces a cell that begins to resemble a spermatozoon (fig. 12, 13).

The maturation phase (fig. 14–17b, 42) continues the organization of the cytoplasm around the manchette, and the middle piece becomes evident. The excess cytoplasm is extruded, except for the cytoplasmic droplet that appears on the sperm cells as they are released from the Sertoli cells into the lumen of the seminiferous tubule. Figure 17a illustrates a dorsoventral, or broadside view; figure 17b shows the appearance as seen from a lateral, or side view.

In the epididymal sperm cells, many of the cytoplasmic droplets change to a distal location, and many are lost completely in ejaculated spermatozoa. In mature spermatozoa, as seen in smears of freshly ejaculated semen, the periodic acid-Schiff reaction shows up as a light pink coloration of the anterior two thirds of the head with a darker red line appearing on the anterior border of the head (fig. 26).

Phase Microscope Observations.—Figure 18 illustrates the cytoplasmic coverings of the nucleus of the sperm cell as described by Hancock.[12] Figure 18A is a schematic drawing which shows the parts of the cytoplasmic coverings removed from the nucleus. Figure 18B illustrates the appearance with the postnuclear cap and the inner acrosome in normal position, with the outer acrosome separate. Figure 18C illustrates the intact spermatozoon and the different morphologic features of the cell, including the equatorial segment.

Figure 19 illustrates the appearance of sperm cells of the boar after a period of storage for 48 to 72 hours. The outer acrosome first becomes swollen and later wrinkled (fig. 19A, 19B). Finally, in semen samples kept at room temperature for 72 hours and longer, the outer acrosome becomes completely detached from the sperm head (fig. 19C). Difficulty was encountered in taking photomicrographs of living spermatozoa as seen by phase microscopy. The movement of the spermatozoa made it impossible to get a clear picture. Figure 35 shows two sperm cells which were fixed by mixing freshly collected semen half and half with 10 per cent formalin. The sperm cells were immobilized, but much of the detail in the head region was lost.

Acid Phosphatase.—In the seminiferous tubules, the early spermatids are lightly

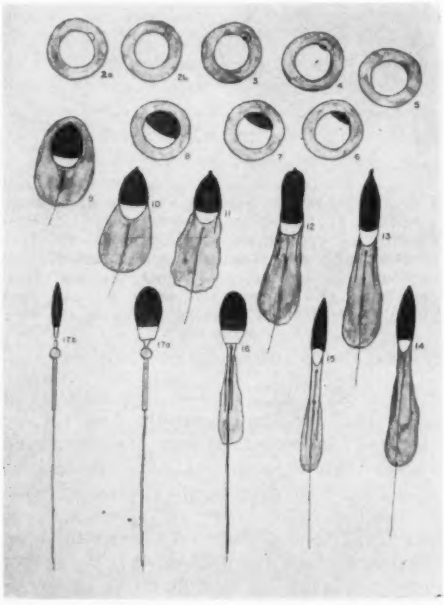

Fig. 2a-17b.—Schematic drawings showing stages of spermiogenesis as followed in PAS preparations. (2a) Early spermatid; (2b) granules become visible in Golgi area; (3) granules coalesce to form acrosome granule; (4) acrosomic vesicle develops; (5) vesicle collapses and granule starts to form cap; (6) cap starts to cover nucleus; (7) cap continues to spread; (8) cap covers half of nucleus; (9) cap covers two thirds of nucleus; (10) tail is evident, nucleus is no longer covered by cytoplasm; (11) tail differentiates further; (12) manchette becomes evident, head takes on sperm shape; (13) acrosome is differentiated; (14) cytoplasm disappears from spermatid; (15) manchette area becomes narrower; (16) further loss of cytoplasm; (17a) mature spermatozoa ready to be released; (17b) lateral view of mature spermatozoa as found free in seminiferous tubule.

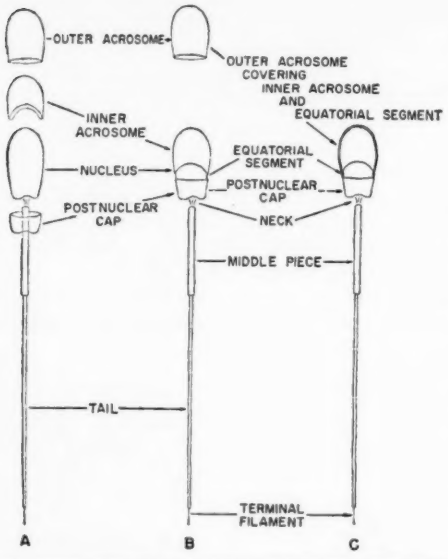

Fig. 18—Schematic drawing showing cytoplasmic coverings of the sperm nucleus. (A) Projected drawing of cytoplasmic coverings removed from nucleus; (B) appearance of sperm nucleus when covered only by the postnuclear cap and inner acrosome; (C) appearance when all the cytoplasmic coverings are in place, including the outer acrosome.

colored after 48 hours of incubation (fig. 44). The acrosomic granule and the developing acrosome appear as structures darker than adjacent material. A dark reacting area surrounds the spermatid when it reaches that stage of spermiogenesis where the head is directed toward the basement membrane and embedded in the Sertoli cell cytoplasm. It is difficult to ascertain whether the reaction is in the spermatid itself or in the Sertoli cell cytoplasm.

After the definitive spermatozoa are released from the Sertoli cells and are free in the lumen, many of them have a marked reaction for acid phosphatase involving the entire sperm cell. Others have the reaction to a lesser extent, with the inner acrosome area reacting most distinctly.

Spermatozoa smears made from material taken from the epididymis have a variety of reactions, ranging from no reaction to a positive reaction of the entire sperm cell. Between these extremes, the inner acrosome areas of some spermatozoa reacted more distinctly than the postnuclear cap. In others, the postnuclear cap reacted most distinctly. In sections of the epididymis, the spermatozoa reacted more noticeably when massed together. Isolated individual spermatozoa seemed to be uniformly darkened, including the cytoplasmic droplet.

Smears made from ejaculated semen had a faint reaction, most predominant in the postnuclear cap (fig. 21); however, some had the most distinct reaction in the inner acrosome area (fig. 20). Reactions were most distinct in smears made from fresh semen. In semen stored for 120 hours, no reaction was evident. Smears prepared from spermatozoa which had been washed free of seminal plasma by repeated centrifugation were nonreactive. Areas of dried seminal plasma on the slide reacted quite strongly (fig. 20–22).

Alkaline Phosphatase.—In the seminiferous tubule, the developing acrosome of the early spermatids reacts very distinctly to the technique for the demonstration of alkaline phosphatase activity. The reaction is

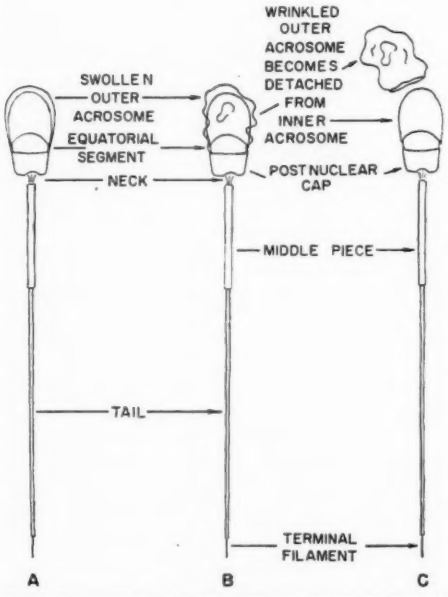

Fig. 19—Schematic drawing illustrating the appearance of some spermatozoa in semen that has been stored for 96 to 120 hours. (A) Outer acrosome becomes swollen; (B) outer acrosome continues swelling and becomes wrinkled; (C) wrinkled outer acrosome becomes detached completely.

more intense in the later spermatids which are intimately related to the Sertoli cells (fig. 43). After the definitive spermatozoa become detached and are free in the lumen of the tubule, the inner acrosome area reacts most distinctly.

In the epididymis, the inner acrosome area is most reactive. The postnuclear cap, middle piece, cytoplasmic droplet, and tail react to a lesser degree. Smears made from epididymal fluid contained spermatozoa which did not react as intensely as those in the sections of the epididymis. Spermatozoa observed in sections of the ductus deferens were faintly reactive, with little difference between the inner acrosome area and other parts of the sperm cell.

Spermatozoa observed on smears made from ejaculated semen reacted distinctly in the inner acrosome area (fig. 23). This reaction was much more distinct than that observed for acid phosphatase. Spermatozoa in smears made from semen stored over 72 hours had a faint reaction or no reaction at all. In smears made from freshly ejaculated semen, it appeared that the reaction was most distinct in areas on smears where there was an accumulation of dried seminal plasma (fig. 23). These granular aggregations reacted quite strongly. Smears prepared from spermatozoa which had been separated from the seminal plasma by repeated washing and centrifuging reacted very faintly or not at all.

Sudan Black B.—In the seminiferous tubules, the middle piece of the late spermatid reacts positively for phospholipid. Mature spermatozoa which are free in the lumen have the same reaction. Epididymal and ejaculated spermatozoa had a similar reaction (fig. 24). There was no evident variation in the amount of reaction in sperm cells studied in semen that had been stored for a long time.

Gomori's Silver Reticulum Stain.—In the seminiferous tubules, the heads of the spermatozoa embedded in the Sertoli cell cytoplasm stained uniformly a dark purple or brown (fig. 45, 46). Spermatozoa in the epididymis stained in a similar way, but the reaction was not as dark.

In smears of the epididymal spermatozoa, the postnuclear cap had the most impregnation.

In smears of ejaculated semen, the postnuclear cap was most definitely impregnated (fig. 25). Most spermatozoa smears reacted similarly, regardless of storage time. One smear, made after 98 hours, had uniform impregnation throughout the heads.

Trichrome Stain.—In all smears prepared with this technique, spermatozoa stained the same, regardless of origin or age of the material. The heads stained blue, with the postnuclear cap slightly darker than the acrosome. The tail and middle piece stained a bright red (fig. 27).

Feulgen Reaction.—In the early spermatids, DNA granules are loosely distributed throughout the nucleus. As spermiogenesis progresses, the granules seem to condense into a homogeneous mass contained within the head of the sperm cell. The body and tail do not react (fig. 28). In spermatozoa from ejaculated semen, the posterior third of the head (postnuclear cap region) stains more intensely than the anterior two thirds of the head. No changes occurred in the staining reaction during storage or aging.

Methyl Green and Pyronine.—The cytoplasm of the early spermatids contains considerable ribonucleic acid (RNA) as revealed by this staining technique. As the spermatids undergo spermiogenesis, the RNA is extruded with the cytoplasm as residual bodies. No RNA was observed in the mature sperm cells.

Casarrett Stain.—With the Casarrett staining technique, the entire spermatozoa is stained pink. The postnuclear cap area stains more deeply than the inner acrosomal area and equatorial segment (fig. 29).

Baker's Hematin.—The middle piece reacts most intensely. The postnuclear cap reacts somewhat, but not as much as the middle piece (fig. 30). Control smears prepared by pyridine extraction had a very slight reaction (fig. 31). When this technique was used, the reactions were not regular and varied to a great extent from one trial to the next. The erratic nature of the results made it difficult or impossible to arrive at any definite conclusions.

Aceto-Carmine Stain.—Aceto-carmine staining differentiates the inner acrosome area, the equatorial segment, and the postnuclear cap. The reaction is the same regardless of the origin or age of the spermatozoa. The inner acrosome area is light pink; the equatorial segment darker pink; and the postnuclear cap is deep red. The middle piece and tail are stained about as intensely as the equatorial segment (fig.

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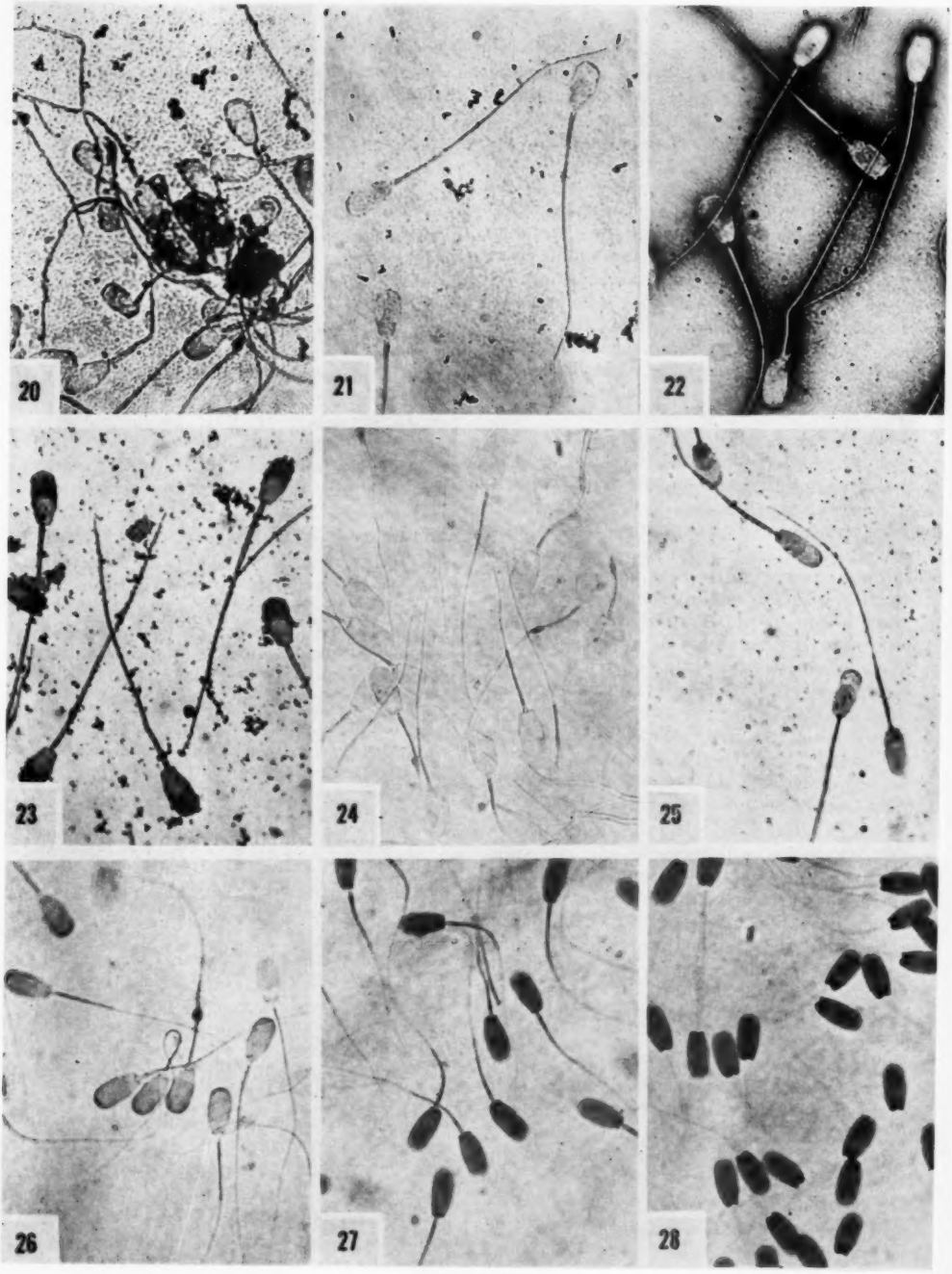

Figures 20-28

32). This technique revealed the most detailed sperm cell morphology and was the easiest to use.

Gram's Stain.—The postnuclear cap stained a darker blue than the inner acrosome area and the equatorial segment (fig. 33). The tail stained red. The posterior margin of the head was the darkest, similar to the reaction with hematoxylin and eosin stain.

Hematoxylin and Eosin.—Smears prepared with the hematoxylin and eosin technique have very few details of the sperm cell structure (fig. 34). The head is blue and the tail and middle piece are red. Smears taken from the head of the epididymis contained many sperm cells with the proximal cytoplasmic droplet evident. In the tail of the epididymis, more were seen with the distal droplet. Ejaculated semen contained a few sperm cells which had the distal droplet. The off-center attachment between the middle piece and head was also quite apparent in all preparations.

Bodian's Protargol.—In the seminiferous tubules, the heads of the developing spermatids appear brown where they were embedded in the Sertoli cell cytoplasm. Mature spermatozoa which are free in the lumen appear to be uniformly stained.

The sperm cells in the lumen of the epididymal tubule sections were uniformly darkened. Spermatozoa in smears made from the epididymal fluid reacted most distinctly in the postnuclear cap. In smears made from ejaculated semen, the majority of the spermatozoa reacted in the postnuclear cap (fig. 37). This was quite regular, regardless of the time after ejaculation. Occasionally the reaction was strongest in the inner acrosome area (fig. 36).

Glycogen Staining.—There were a few fine granules of glycogen around the clusters of spermatid heads embedded in the cytoplasm of the Sertoli cells. No evidence of glycogen was observed in the definitive spermatozoa after they were released into the lumen of the seminiferous tubules.

In the epididymis, the spermatozoa had no evidence of glycogen. However, the cytoplasm of the epididymal epithelial cells contained many glycogen granules.

Spermatozoa in smears of epididymal fluid were negative for glycogen. All ejaculated spermatozoa were negative for glycogen by either the Bauer-Feulgen or PAS techniques.

Discussion

According to Hancock,[12] the anterior two thirds of the head are covered by a cytoplasmic cap, the acrosome. He distinguished two different parts of the acrosome, the outer acrosome and the inner acrosome. The outer acrosome is larger and its posterior border is straight and coincides with the anterior border of the postnuclear cap which covers the posterior third of the head (fig. 18a, 18b). Hancock described the inner acrosome as a crescentic structure with the concave border directed caudally and its convex border following the outline of the front of the head. The area bounded by the concave posterior border of the in-

Legends for Figures 20-28 on Opposite Page

Fig. 20—Smear of fresh boar semen fixed in acetone and incubated 72 hours for acid phosphatase. x 895.

Fig. 21—Smear of boar semen stored 24 hours at 3 C., fixed in acetone, and incubated 72 hours for acid phosphatase. x 895.

Fig. 22—Smear of boar semen stored 96 hours at 3 C., fixed in acetone, and incubated 48 hours. Acid phosphatase technique; x 895.

Fig. 23—Smear of boar semen stored three hours at 3 C., fixed in chilled alcohol, and incubated four hours. Alkaline phosphatase technique; x 895.

Fig. 24—Smear of fresh epididymal fluid fixed in 10 per cent formalin. Sudan black B.; x 895.

Fig. 25—Smear of washed spermatozoa from semen stored 24 hours at 3 C.; fixed in 10 per cent formalin. Gomori's silver stain; x 895.

Fig. 26—Smear of washed boar spermatozoa from fresh semen; fixed in Zenker's formal. PAS; x 895.

Fig. 27—Smear of fresh boar semen fixed in 10 per cent formalin. Trichrome stain.

Fig. 28—Smear of washed spermatozoa from fresh boar semen fixed in 10 per cent formalin. Feulgen test for DNA; x 895.

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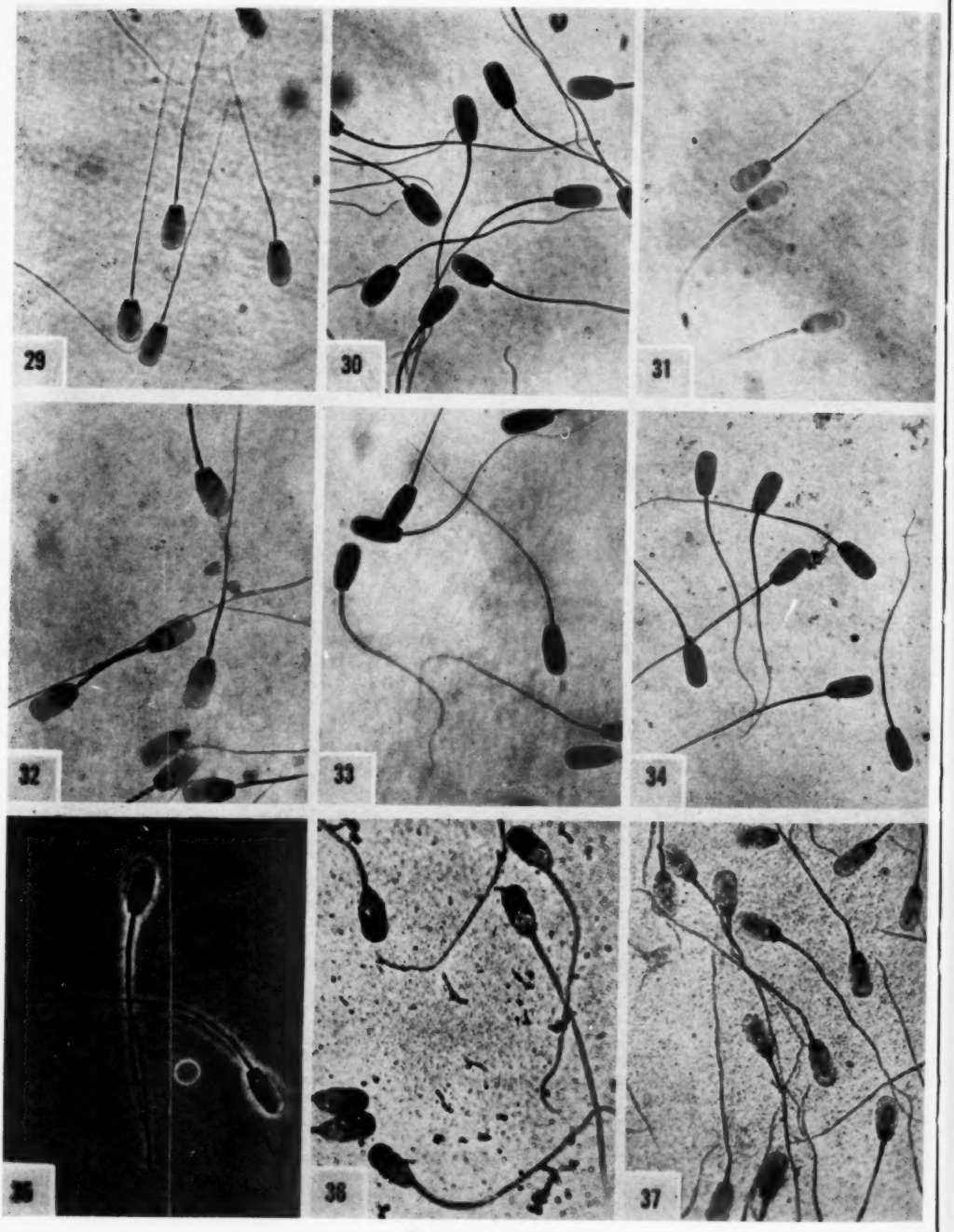

Figures 29-37

ner acrosome and the straight posterior margin of the outer acrosome is called the equatorial segment. He described the concave posterior border of the head where the three neck granules are located at an off-center position. The neck fibers are connected to the granules and attach the head to the middle piece (fig. 18e). The findings of this study agree with the descriptions made by Hancock.

The PAS staining technique does not differentiate between the inner and the outer acrosome. It may be that only the outer acrosome reacts, since the equatorial segment stains with the same intensity as the inner acrosome area. One could conclude from these staining reactions that the outer acrosome contains mucopolysaccharides and mucoproteins that are not present in the postnuclear cap. It has been postulated that the outer acrosome is an important structure for the role of fertilization of the ovum. If this structure deteriorates and is lost, as was observed with the phase microscope, it may render the spermatozoon incapable of fertilizing an ovum.

The separation and wrinkling of the outer acrosome, observed in spermatozoa that had been stored for a period of time, indicate that degenerative processes may occur due to enzymatic or bacterial action. After 24 hours of storage, a great number of motile bacteria could be observed in the seminal fluid. The effect that the metabolic by-products of microorganisms may have on spermatozoal metabolism is an important problem which should be studied further.

The significance of the presence of acid and alkaline phosphatase in the Sertoli cells, in the epididymis, and in ejaculated semen is a matter of conjecture. It would seem from the observations made in this work that these enzymes are not incorporated inside the sperm cell itself, but rather are outside the boundaries of the cell. Possibly the enzymes are bound to the surface of the cell by adsorption where the enzymatic activity takes place, or perhaps the enzyme is very superficially located in the substance of the sperm cell. The reaction of spermatozoa, which had been repeatedly washed and centrifuged, was weak or negative. The enzyme was either washed from the surface or leached from the substance of the sperm cell. Areas of dried seminal plasma on slides prepared from whole semen are definitely reactive to the phosphatase technique. It would seem that there is a generous quantity of acid and alkaline phosphatase in the seminal fluid.

Phosphatase is one of a group of general classifications of enzymes and its activity does not necessarily involve any specific sugar or carbohydrate. The function of catalyzing the hydrolysis of hexose phosphate to phosphoric acid and the free sugar, as occurs in the transfer of sugars such as glucose 6-phosphate from the epithelial cells of the intestine to the blood glucose in the capillaries of the intestinal villi, was shown by Dempsey and Deane.[7] Possibly a similar situation exists with the developing spermatid and mature sperm cell where hexose phosphate is hydrolyzed by phosphatase in the metabolism of the sperm cell or spermatid.

On smears treated with the phosphatase stains, the location of the reaction in the

Legends for Figures 29-37 on Opposite Page

Fig. 29—Smear of fresh boar semen fixed in 10 per cent formalin. Casarrett stain; x 895.

Fig. 30—Smear of fresh boar semen fixed in formal calcium. Baker's hematin; x 895.

Fig. 31—Smear of fresh boar semen fixed in weak Bouin's solution. Baker's hematin stain; x 895.

Fig. 32—Smear of fresh boar semen stained and fixed in aceto-carmine solution. x 895.

Fig. 33—Smear of washed spermatozoa from boar semen stored for 24 hours; fixed in 10 per cent formalin. Gram stain; x 895.

Fig. 34—Smear of washed spermatozoa from fresh boar semen fixed in 10 per cent formalin. H & E stain; x 895.

Fig. 35—Wet preparation of freshly killed spermatozoa in 10 per cent formalin, 24 hours after fixation. Photomicrograph taken through a phase microscope. x 895.

Fig. 36—Smear of fresh boar semen fixed in alcohol acetic acid and formalin. Protargol stain; x 895

Fig. 37—Smear of fresh boar semen fixed in alcohol acetic acid and formalin. Protargol stain; x 895.

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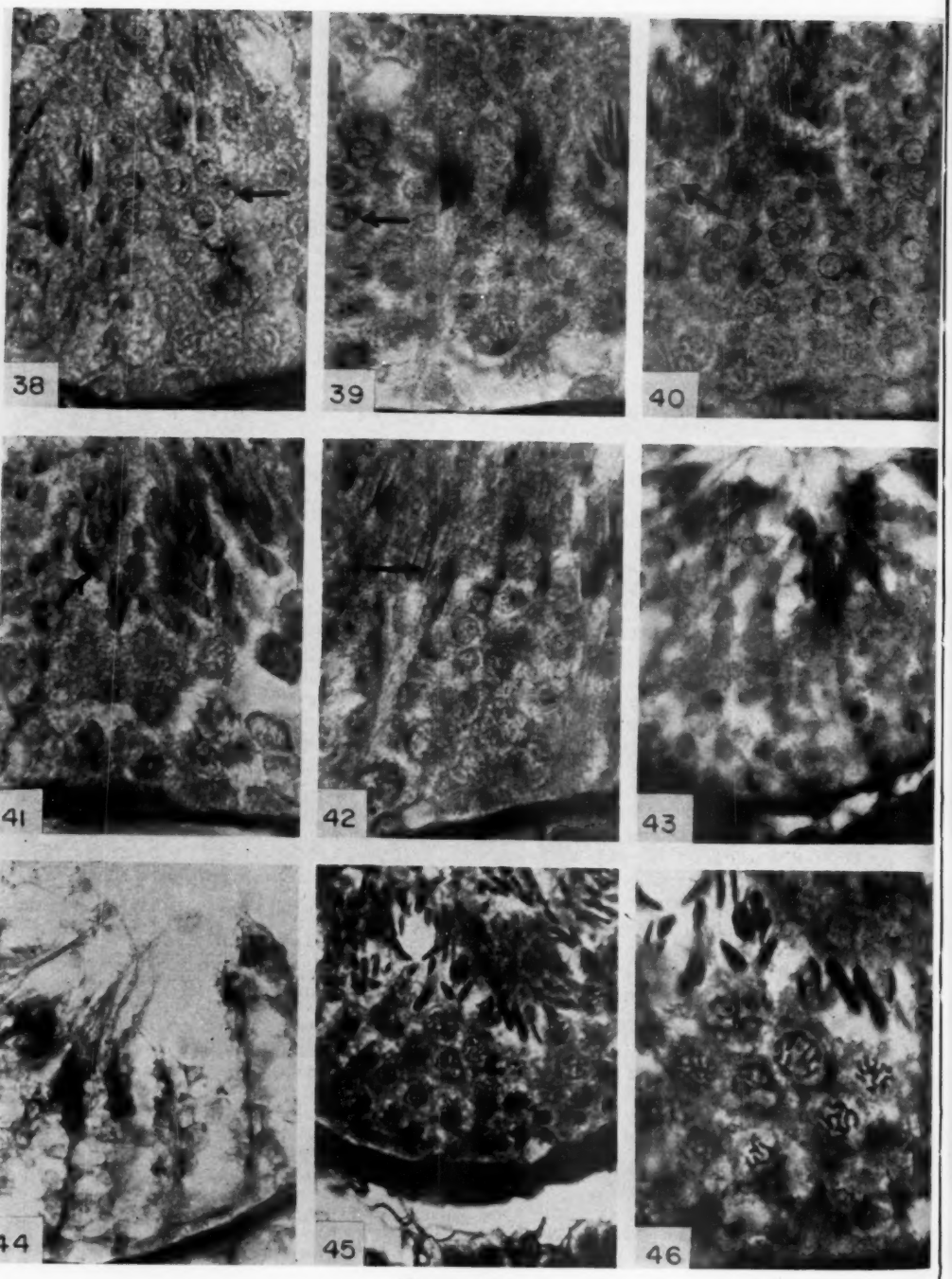

Figures 38-46

sperm cell varies. With the acid phosphatase technique, some spermatozoa react most distinctly in the inner acrosomal area; others react in the postnuclear cap region. With alkaline phosphatase, the inner acrosomal area of the spermatozoa is definitely most reactive, particularly in freshly ejaculated semen. The fact that clumps of spermatozoa near an aggregation of dried seminal plasma react most distinctly would support the hypothesis that the seminal plasma contained the enzyme rather than the sperm cell itself. The use of phosphatase studies on semen smears would seem to have limited value in obtaining information about the condition and integrity of spermatozoa after storage. There is a great amount of variation in the reaction produced. This variation seems to be more dependent upon the amount of seminal plasma included on the smear than upon the metabolic condition of the sperm cell.

The reaction observed with Sudan black B technique when used on mature spermatozoa indicates that the middle piece of the sperm cell contains considerable phospholipid or lipids other than triglycerides. These results are similar to the findings of Melampy *et al.*,[19] who studied bovine material.

The Feulgen technique produced a positive reaction throughout the head and there was a slight line of demarcation between the outer acrosome and the postnuclear cap. The posterior third of the nucleus reacted more strongly than the anterior two thirds of the head. This could be interpreted as indicating a higher concentration of DNA in the postnuclear cap area.

After methyl green and pyronine staining, the residual bodies stained a deep red. After treatment with ribonuclease, these bodies were not evident. This would lead to the conclusion that the residual bodies observed in the lumen of seminiferous tubules containing late spermatids represent RNA being extruded with the cytoplasm of the spermatid. The mature spermatozoa contain no detectable RNA. This is consistent with the theory that the endoplasmic reticulum and microsomes of the spermatid are discarded and make no contribution to the zygote at the time of fertilization. Most descriptions of spermiogenesis fail to explain what becomes of the microsomes and endoplasmic reticulum of the early spermatid. With the techniques used, there was no structure in the definitive sperm cell which could be traced back to these cytoplasmic organoids.

No glycogen was observed in any sperm cells by either the PAS reaction or the Bauer-Feulgen technique. This is similar to the results reported for the bull by Melampy *et al.*[19]

The postnuclear cap of the spermatozoa studied had greater affinity for silver impregnation than any other part. It was difficult to interpret the results of the silver impregnation and the protargol techniques. Why certain parts of the sperm cell were impregnated more than others is not known. It is likely that, as suggested by Melampy *et al.*,[19] the coverings of the different parts of the sperm cell possess dissimilar molecular constitutions and, therefore, may have different adsorptive capacities for various environmental constituents such as enzymes and substrates.

The gradual disappearance of the cyto-

Legends for Figures 38-46 on Opposite Page

Fig. 38—Boar testis stained with PAS. Arrow indicates spermatid in Golgi phase with acrosomic granule.

Fig. 39—Boar testis stained with PAS. Arrow indicates spermatid in Golgi phase with acrosomic vesicle. x 895.

Fig. 40—Boar testis stained with PAS. Arrow indicates spermatid in cap phase.

Fig. 41—Boar testis stained with PAS. Arrow indicates spermatid in acrosome phase.

Fig. 42—Boar testis stained with PAS. Arrow indicates spermatid in maturation phase. x 895.

Fig. 43—Boar testis fixed in 80 per cent alcohol and incubated for four hours by the alkaline phosphatase technique. x 495.

Fig. 44—Boar testis fixed in acetone and incubated for 48 hours by the acid phosphatase technique. x 495.

Fig. 45—Boar testis fixed in Rossman's fluid and impregnated with Gomori's silver stain. x 495.

Fig. 46—Boar testis fixed in Rossman's fluid and impregnated with Gormori's silver stain. x 895.

plasmic droplet observed as the spermatozoa progress through the epididymis is in agreement with the report of Rao and Berry.[21]

SUMMARY

Semen smears were studied from 5 Yorkshire boars which previously had been proved fertile. Smears were made from fresh semen as well as semen that had been stored up to 120 hours. Unstained living and dead spermatozoa were studied in wet preparations by means of phase microscopy. After the studies on ejaculated semen were completed, the boars were castrated. Immediately after castration, smears were made of fresh epididymal fluid collected from the epididymis. Tissue blocks were collected from various areas of the testicle, epididymis, and ductus deferens for histologic studies. Histologic sections made from the tissue blocks and smears were stained by means of cytochemical and histochemical techniques. The following findings merit emphasis.

1) No glycogen was demonstrated in the mature sperm cells of the boars studied.

2) Sudanophilic phospholipids were present in the middle pieces and cytoplasmic droplets. No variation was observed with aging.

3) Mucopolysaccharides were demonstrated in the outer acrosome of developing and mature spermatozoa.

4) The entire nuclear head of the sperm cells studied reacted to the Feulgen test. The strongest reaction occurred in the posterior third of the nuclear head.

5) No ribonucleic acid was demonstrated in the mature sperm cells of the boars studied.

6) Cytoplasmic droplets were observed in sperm cells in ejaculated semen. Sperm cells obtained from the head of the epididymis had the greater percentage of such droplets. Samples collected from the tail of the epididymis had fewer spermatozoa with proximal droplets and a greater percentage with distal cytoplasmic droplets.

7) The reaction of porcine spermatozoa for the phosphatase enzymes was extremely variable. This variation seemed to be more related to the enzymes in the seminal plasma than the enzyme content of the sperm cells.

8) Histochemical and cytochemical reactions seem to be of questionable value in determining the metabolic state or degree of exhaustion of porcine spermatozoa.

9) Degenerative changes of spermatozoa after 72 to 96 hours of storage were observed by means of the phase microscope. As the semen sample became older, a greater percentage of the spermatozoa underwent changes in which the outer acrosome became enlarged and swollen. Later, the swollen outer acrosome became wrinkled. As the semen sample aged further, a great percentage of the spermatozoa in the sample lost the outer acrosome completely from the inner acrosome and nucleus.

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SUMMARIO IN INTERLINGUA

Reactiones Cytochimic de Porcin Spermatidas e Spermatozoides

Frottis de semine esseva studiate ab 5 verres Yorkshire de previemente provate fertilitate. Frottis esseva facite de semine fresc e etiam de semine preservate durante usque a 120 horas. Non-tincturate spermatozoides vive e morte esseva studiate in preparatos humide per medio de microscopia a differentia de phase. Post le completion del studios in le ejaculate semine, le verres esseva castrate. Immediatemente post le castration, frottis esseva facite de fresc liquido epididymal colligite ab le epididymide. Blocos de tissu esseva obtenite ab varie areas del testes, del epididymide, e del ducto deferente pro studios histologic. Sectiones histologic facite ex le blocos de tissu e frottis esseva tincturate per medio de technicas cyto- e histochimic. Le sequente resultatos merita esser reportate.

1. Nulle glycogeno esseva demonstrate in le spermatozoides matur del verres studiate.

2. Phospholipidos sudanophile esseva presente in le pecias intermedie e in le guttettas cytoplasmatic. Nulle variationes esseva notate in correlation con le etate del verres.

3. Mucopolysaccharidas esseva demonstrate in le acrosomas exterior de spermatozoides in disveloppamento e de spermatozoides matur.

4. Le complete capite nuclear del cellulas spermatic studiate reageva al test de Feulgen. Le reaction le plus forte occurreva in le tertio posterior del capite nuclear.

5. Nulle acido ribonucleic esseva demonstrate in le spermatozoides matur del verres studiate.

6. Guttettas cytoplasmatic esseva observate in cellulas spermatic del ejaculate semine. Cellulas spermatic obtenite ab le capite del epididymide habeva le plus grande procentage de tal guttettas. Specimens colligite ab le cauda del epididymide habeva minus spermatozoides con guttettas proximal e un plus grande procentage con distal guttettas cytoplasmatic.

7. Le reaction de spermatozoides porcin pro le enzymas de phosphatase esseva extrememente variabile. Iste variation pareva esser relationate plus al enzymas in le plasma seminal que al contento de enzymas in le cellulas spermatic.

8. Reactiones histo- e cytochimic pare esser de questionabile valor in determinar le stato metabolic o le grado de exhaustion de spermatozoides porcin.

9. Alterationes degeneratori in le spermatozoides preservate durante 72 a 96 horas esseva observate per medio del microscopio a differentia de phase. Con le vetulation del specimen de semine, un crescente numero de spermatozoides suffreva alterationes in que le acrosomas exterior deveniva allargate e tumide. Plus tarde le tumide acrosomas exterior deveniva rugate. In le curso del subsequente vetulation del specimens de semine, un alte procentage de spermatozoides perdeva completemente le acrosoma exterior ab le acrosoma interior e le nucleo.

XUM

Immunity and the Reproductive Tract of Laying Hens Vaccinated with the Tissue Culture Newcastle Disease Vaccine

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PREVIOUS STUDIES on tissue culture-modified Newcastle disease (TCND) vaccine have been conducted under experimental or isolated conditions in which immunity of birds was challenged by intramuscular (i.m.) inoculation or by exposure of principals to a mist of virus produced by a DeVilbiss No. 40 atomizer.[3,4] Neither route measured the immune response accurately. The unnatural i.m. route was less sensitive than the airborne route of exposure in detecting an immunity that would protect against a decrease in egg production. The airborne route resulted in isolation of the virus from the respiratory tract, although the systemic and reproductive tracts were refractory to the virulent virus. Since these artificial methods of challenge were manifestly deficient, vaccinated birds and unvaccinated pen contact controls on commercial poultry farms lacking an adventitious infection with Newcastle disease were exposed in a confined area to susceptible chickens infected intratracheally with the virulent GB-Texas-1946 strain of Newcastle disease virus (NDV).

MATERIALS AND METHODS

Vaccination of Chickens.—A replacement flock of 28,000 chickens was built by obtaining 3,500 1-day-old chicks at two-week intervals. The chicks were placed in a thoroughly clean brooder house composed of five pens, each pen housing a fifth of the brood. When the birds were 9 weeks old, it was customary to allot those in each pen to two groups, removing one-half of the chicks to similar pens in another brooder house.

The vaccination schedule included infectious bronchitis (IB) vaccine given as a dust to chickens 2 to 2½ weeks old and repeated when the chickens were 13 to 15 weeks old; however, the second IB vaccination was always given seven to ten days before or after the second dose of the Newcastle disease (ND) vaccine. The fowlpox and laryngotracheitis vaccines were administered simultaneously when chickens were 8 weeks of age. Vaccination against ND consisted of two doses of the TCND vaccine; the first was given to 5- to 6-week-old birds and the second was given when the chickens were 13 to 14 weeks of age, at a time when they were handled for some other operation, such as specking or change of quarters.

Birds in all eight lots were vaccinated and carefully observed; those in lots 1 and 2 (DeKalb 101 chickens) were studied in detail. Careful observations were made regarding condition, health, and feed consumption, and blood samples were obtained at frequent intervals for serologic study. The chickens were removed to the laboratory for immunity challenge.

Control chickens for study of the spread of the vaccine virus and detection of an adventitious infection were 30 unvaccinated birds in each pen of vaccinated birds. In all instances, the control chickens were in the pen during the vaccination procedure.

Tissue Culture Vaccine Virus.—The vaccine virus for the eight lots was supernatant fluid of the seventeenth to twentieth passages of NDV propagated in minced chicken embryo tissue.[5] Just before use, concentrated supernatant virus suspension of a known titer was diluted with cold, buffered, NaCl solution to an embryo infectivity titer of $10^{4.5}$ per 0.2 ml. Penicillin and streptomycin at the respective rates of 2,000 and 1,000 units per 100 ml. of vaccine were incorporated to control contamination. The dose of vaccine given to each chicken was 0.2 ml. injected into the breast muscle.

Serology.—The procedures used in the hemagglutination-inhibition (HI) and serum neutralization (SN) tests have been described.[5] Samples of blood were taken from 50 vaccinated and 30 control chickens picked at random four, eight, 12, 24, 32, 44, 59, 74, and 89 weeks after vaccination (table 1). The HI tests were conducted on all individual samples. The SN titers were obtained in 20 serum samples selected for uniform distribution throughout the range of HI titers obtained. The SN titers of the controls were obtained from ten samples picked at random, since none of the controls was found to have an HI titer.

Method of Challenge.—Challenge consisted of placing 8 chickens on a litter of shavings in an enclosed unit measuring 4 by 5 by 7 ft. When the layers became acclimatized and reached a stable

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level of egg production in the new environment (18 to 24 days), 2 susceptible chickens were inoculated intratracheally with 0.2 ml. of NDV (strain GB-Texas; titer, 5 x 10^7 embryo m.i.d./ml.) and immediately placed in the unit as pen contacts. They constituted infected penmates; a relatively crowded area insured close cohabitation. Observations and daily egg production records were kept for at least 28 days after exposure.

Intramuscular (i.m.) challenge with the GB strain of NDV was conducted as previously described.[5]

RESULTS

No signs of ND were observed in any of the chickens after vaccination at 5 to 6 weeks of age or following the second dose at 13 to 14 weeks of age. The chickens of lot 1 that were given IB vaccine at 2½ weeks of age had a delayed reaction. No signs were observed until the twelfth postvaccination day, when coughing, sneezing, and a drop in feed consumption were observed and respiratory distress was obvious. Moderate to abundant amounts of a seromucous type of tracheal exudate were obtained at necropsy, and the virus was isolated. In spite of the respiratory distress due to IB, the principals were vaccinated with the TCND vaccine as scheduled; the respiratory

TABLE 1—Hemagglutination-Inhibition Titers in Four Groups of Chickens (Two Lots) Given Tissue Culture-Modified Newcastle Disease Vaccine and Exposed by Contact to Chickens Infected with the Texas-GB Strain of Newcastle Disease Virus

	Vaccinated chickens				Control chickens			
	Contact exposure		i.m. exposure		Contact exposure		i.m. exposure	
Group No.	No. of birds	Titer increase	No. of birds	Titer increase	No. of birds	Titer increase	No. of birds	Titer increase
LOT 1—BIRDS CHALLENGED WHEN 83.4 WEEKS OLD								
1	1	0–0	1	0–40	2	0–320 or > (signs but recovered)	2	DBS*
	1	0–10	1	0–80				
	1	10–0	2	0–160				
	1	10–20	1	10–160	5	DBS		
	1	20–40	2	0–320 or >				
	1	10–80	1	10–320 or >				
	1	0–80	1	40–320 or >				
	1	0–320 or >						
2	1	0–160	1	10–80	5	0–320 or > (signs but recovered)	2	DBS
	1	10–160	3	0–320 or >				
	2	0–320 or >	3	10–320 or >				
	2	10–320 or >	3	20–320 or >				
	2**	20–320 or >						
3	1	0–0						
	1	0–40						
	1	10–40						
	2	10–80						
	1	0–320 or >						
	2	10–320 or >						
4	1	0–160						
	1	0–320 or >						
	5	10–320 or >						
	1	20–320 or >						
LOT 2—BIRDS CHALLENGED WHEN 101 WEEKS OLD								
1	1	10–80	1	0–40	4	0–320 or > (3 paralyzed, 1 refractory)	1	0–320 or >
	3	0–320 or >	1	0–80				
	1	10–320 or >	1	10–160				
	2	20–320 or >	1	0–320 or >				
	1	80–320 or >	2	10–320 or >	4	DBS	7	DBS
			2	20–320 or >				
2	2	0–160	3	0–320 or >				
	4	0–320 or >	5	10–320 or >				
	1	10–320 or >			8	DBS		
	1	40–320 or >						
3	3	0–320 or >						
	2	10–320 or >						
	2	20–320 or >						
	1†	DBS						
4	5	0–320 or >						
	2	10–320 or >						
	1	40–320 or >						

* Died before date blood samples were obtained. ** One bird had slight râles and depression on only the twelfth day after challenge. † Died from causes other than Newcastle disease; egg-bound one day prior to challenge.

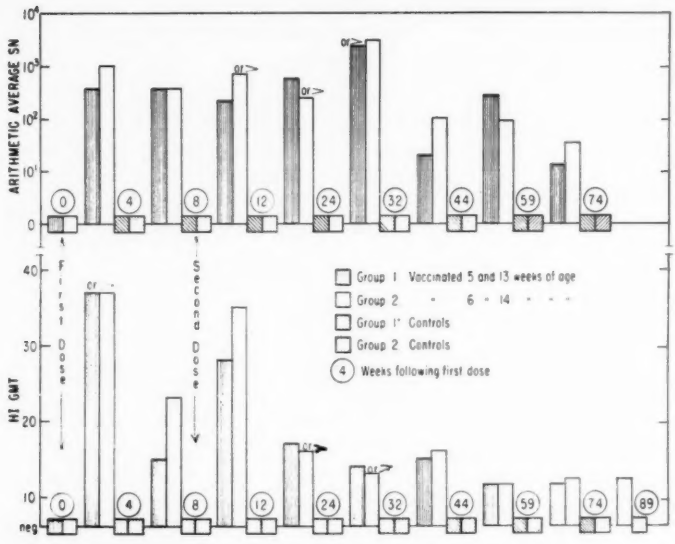
ARITHMETIC AVERAGE SN
HI GMT
First Dose
Second Dose
Group 1 Vaccinated 5 and 13 weeks of age
Group 2 " 6 " 14 " " "
Group 1 Controls
Group 2 Controls
(4) Weeks following first dose

Fig. 1—Serologic response in two flocks of chickens vaccinated with tissue culture-modified Newcastle disease vaccine.

signs continued for 48 hours, and recovery was then rapid, with no losses due to the IB vaccine reaction. Thus, the TCND did not appear to aggravate the respiratory signs, since the birds continued a steady increase in feed consumption. During the three weeks after TCND vaccination, 22 chickens died from causes not attributed to IB or ND.

The principals of lot 2 had a delay of nine days in reacting to the IB vaccine. The respiratory distress, decrease in feed consumption, and slight depression reached their peak more rapidly than in lot 1, and recovery was apparent by the time they were vaccinated with the first dose of TCND vaccine, at six weeks of age. Mortality dur-

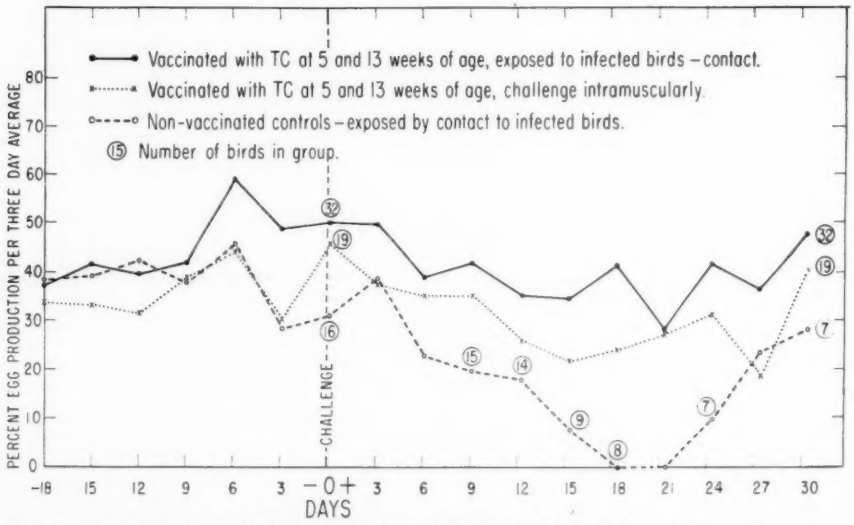
Vaccinated with TC at 5 and 13 weeks of age, exposed to infected birds – contact.
Vaccinated with TC at 5 and 13 weeks of age, challenge intramuscularly.
Non-vaccinated controls – exposed by contact to infected birds.
(15) Number of birds in group.
PERCENT EGG PRODUCTION PER THREE DAY AVERAGE
CHALLENGE
DAYS

Fig. 2—Egg production of 83-week-old, tissue culture-vaccinated chickens after contact exposure and intramuscular challenge with the Texas-GB strain of Newcastle disease virus.

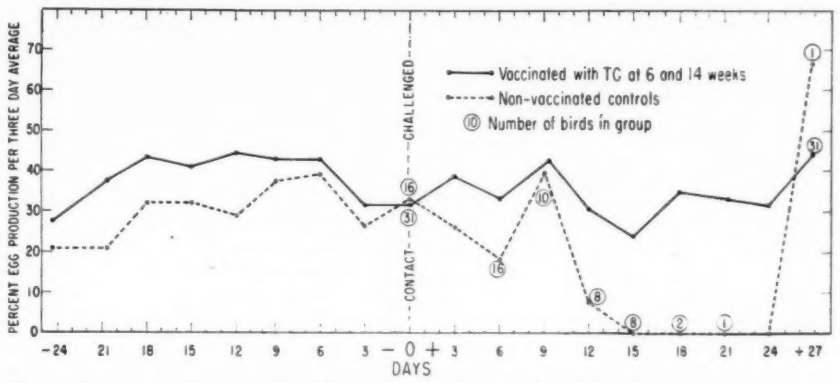

Fig. 3—Egg production of 101-week-old, tissue culture-vaccinated chickens after contact exposure to birds infected with the Texas-GB strain of Newcastle disease virus.

ing the first three weeks after vaccination was 27 of 3,500 chickens (0.77%); causes were unknown.

Serologic Response to TCND Vaccination.—The serologic response of the serums collected at intervals from birds in lots 1 and 2, beginning four weeks after the first dose of vaccine and continuing through their productive life, is illustrated (fig. 1). The HI response to the first dose of vaccine was significant in both groups. The titers decreased by the eighth week. A second peak followed the second dose, and the HI titers receded to values that were relatively low but significant, in that some persisted throughout the productive life of the birds. The SN titers responded similarly, but appeared to decrease more slowly and were more stable in view of the fact that serums were collected from birds at random. The SN titers persisted for at least 74 weeks after the first vaccination. The two groups of chickens reacted similarly to the two prepared batches of vaccine in spite of the fact that the age of the birds at the time of vaccination varied by one week. No HI titers or significant SN titers were found in any of the controls during the entire experiment.

During the experiment, 8 chickens were removed from lots 1 and 2 for intramuscular challenge 30, 45, 53, 62, 66, and 72 weeks after the first dose of vaccine. On each occasion, a lesser number of controls were also removed for determination of the immunologic status of the unvaccinated

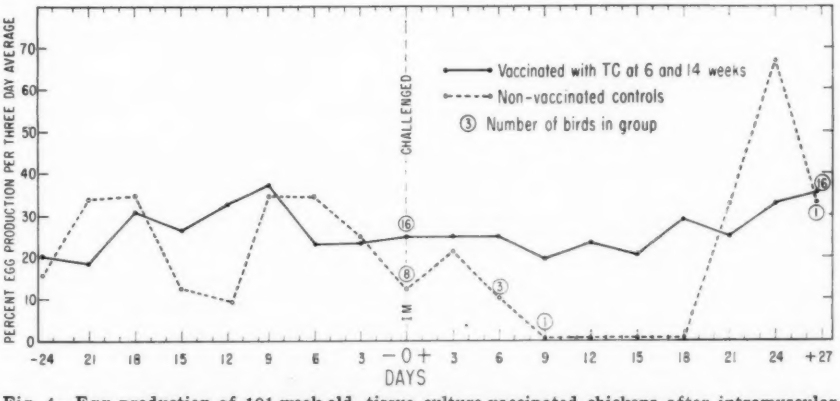

Fig. 4—Egg production of 101-week-old, tissue culture-vaccinated chickens after intramuscular challenge with the Texas-GB strain of Newcastle disease virus.

chickens and for detection of any adventitious exposure that might not have been detected serologically. All 96 vaccinated chickens were refractory and all of 20 controls were susceptible. Investigation of fewer principals obtained at random from lots 3 through 8 (housed in various areas of the ranch during the 46th to 50th weeks after the initial vaccine dose) also indicated that immunity was maintained and adventitious infection had not occurred.

Exposure of Layers by Contact with Infected Chickens.—Since the flock escaped field exposure to ND, the degree of protection that vaccination afforded the reproductive tract at the end of the productive life of the hens was ascertained by artificial means.

Laying Hens of Lot 1.—When the birds were 79 weeks old, 51 vaccinated and 16 control chickens were brought to the University and allotted to groups of 8 each for challenge by the GB strain of ND virus. Figure 2 illustrates egg production of four units, consisting of 32 vaccinated hens, and two units, containing 16 unvaccinated control hens, after exposure to experimentally infected penmates.

The intratracheally inoculated contact chickens generally showed definite and audible râles by the 72nd hour. This was followed by progressive depression, paralysis, and clonic spasms; death occurred on the fifth or sixth day. Occasionally, a completely paralyzed bird lived ten days.

None of the 32 TCND-vaccinated chickens exposed to infected birds showed clinical evidence of ND, except for 1 that had audible râles on the twelfth day. Egg production remained within the limits of normal fluctuation.

Manifestation of disease in the two pens of susceptible controls began with audible râles in 2 birds seven days after exposure. The disease progressed rapidly throughout all the birds, in the form of râles and depression, by the eighth day. On the ninth day, 1 was found dead, and mortality increased progressively. Though all the principals developed clinical signs, 7 recovered. Egg production dropped suddenly on the sixth day, and stopped completely by the fifteenth day. The flock remained unproductive for three additional days. The 7 remaining birds made an apparent clinical recovery.

The high degree of resistance in the vaccinated group was corroborated by i.m. challenge. No respiratory or nervous signs of ND were observed when the immunity of 19 birds was challenged. A slight depression in the egg production curve of the i.m.-vaccinated birds may have been caused by the use of susceptible laying hens as the infected contacts. Eggs laid by these hens before inoculation were included in the pre-challenge figures.

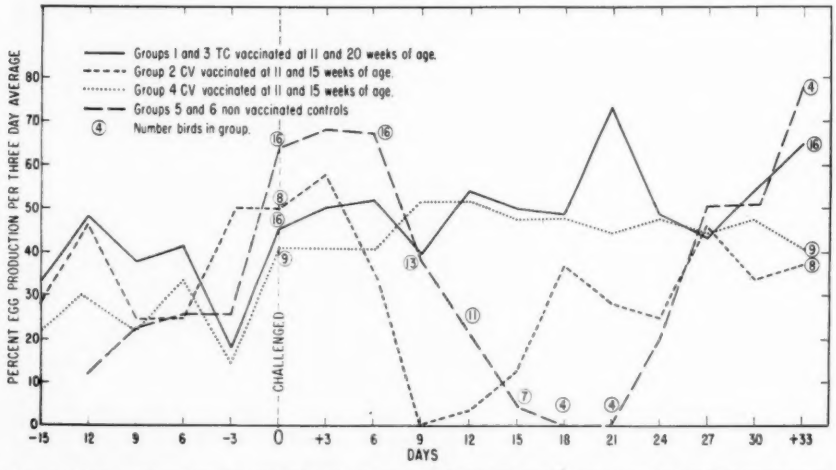
Groups 1 and 3 TC vaccinated at 11 and 20 weeks of age.
Group 2 CV vaccinated at 11 and 15 weeks of age.
Group 4 CV vaccinated at 11 and 15 weeks of age.
Groups 5 and 6 non vaccinated controls
Number birds in group.
PERCENT EGG PRODUCTION PER THREE DAY AVERAGE
CHALLENGED
DAYS
-15 12 9 6 -3 0 +3 6 9 12 15 18 21 24 27 30 +33
0 10 20 30 40 50 60 70 80

Fig. 5—Egg production of 76-week-old tissue culture-vaccinated and crystal violet-vaccinated chickens after exposure to birds infected with the Texas-GB strain of Newcastle disease virus.

To determine if all of the birds were exposed by contact, blood serums collected at the beginning and end of the experiment were tested for HI antibodies. Only 3 vaccinated birds showed no increase in HI titer, whereas 29 showed an increase in titer by 1 to 6 doubling dilutions or greater (table 1).

Laying Hens of Lot 2.—When birds of lot 2 reached 95 weeks of age, 72 hens (48 vaccinated and 24 unvaccinated pen contacts) were removed to isolation quarters.

The immunity of birds in four vaccinated groups and two unvaccinated control groups was challenged when they were 101 weeks old by contact with 2 infected cockerels. The 2 inoculated cockerels in each pen showed signs of râles, depression, and leg weakness by the third or fourth day, and progressively severe signs were followed by death after eight or nine days.

On the seventh day after exposure, the unvaccinated controls showed marked depression and audible, moist râles; 1 hen was partially paralyzed. On the eighth day, depression and respiratory signs continued and the paralyzed hen was dead. On the ninth day, 5 birds had paralysis of the wings and legs, and clonic spasms. Nine birds were depressed and had audible râles, and 1 hen appeared unaffected. A decrease in egg production was observed on the ninth postchallenge day, or two days after the onset of respiratory signs in the unvaccinated controls. One egg was found in the pen of the apparently unaffected hen on the eleventh day, and production then ceased through the 24th day after challenge.

No signs of ND were observed in the four vaccinated groups. Egg production was similar in all four groups (fig. 3). The production curve dropped slightly on the fifteenth postchallenge day, but immediately returned to the higher level.

Figure 4 shows the results of i.m. inoculation of 16 vaccinated chickens and 8 unvaccinated controls. The vaccinated group had no decrease in egg production or other signs of NDV infection. In the susceptible controls, death first occurred on the fourth day after challenge, and 7 of the 8 had succumbed by the ninth day. One that showed marked depression and cessation of egg production by the eighth day recovered clinically and returned to production by the 21st day.

As shown (table 1), all birds whose immunity was challenged by contact or by i.m. inoculation had a rise in HI titer, indicating that exposure to an infection by the virulent virus had been accomplished.

TABLE 2—Hemagglutination-Inhibition Titers Before and After Contact Exposure of 76-Week-Old Vaccinated Chickens

Vaccinated chickens			Control chickens	
Group No.	No. of birds	Titer increase	No. of birds	Titer increase
TISSUE CULTURE VACCINE (LIVE)				
1	1	20–40	4	0–320 or >
	1	40–40		(3—signs but
	1	40–80		recovered)
	1	0–80		1—paralyzed
	1	0–320 or >	4	DBS*
	1	10–320 or >		
	1	20–320 or >		
	1	40–320 or >		
3	2	0–320 or >	3	0–320 or >
	2**	10–320 or >		(1—signs but
	3	20–320 or >		recovered)
	1	40–320 or >		2—paralyzed
			5	DBS
CRYSTAL VIOLET VACCINE (INACTIVATED)				
2	7†	0–320 or >		
	1	10–320 or >		
4	5	0–0		
	2	0–10		
	2	0–320 or >		

* Died before date blood samples were obtained. ** One of these birds had moist râles on the eighth day after challenge. Two of the 8 vaccinated birds had moist râles on the eighth day after challenge.

Exposure of Birds Vaccinated with TCND and Crystal Violet-Inactivated Vaccines.—In a previous study, chickens were vaccinated at 11 and 15 weeks of age with CV and at 11 and 20 weeks with the TCND. Evaluation of immunity by challenge with GB strain of NDV, administered with the DeVilbiss atomizer, proved unsatisfactory for studying the protection offered the reproductive tract.

When the chickens in this experiment were 76 weeks old, the unvaccinated and CV- and TCND-vaccinated birds of the previous study were allotted to groups of 8 per unit,* and each group was exposed to infected cockerels (fig. 5).

Birds in the unvaccinated pen contact control group showed evidence of typical ND by the fourth day. The disease progressed with depression, clonic spasms, cessation of egg production, and death of 9 of 16 birds by the fifteenth day. Four of the birds recovered and resumed production on the 21st day following challenge.

The TCND- and CV-vaccinated birds did

* One group (unit 4) consisted of 9 birds vaccinated with CV.

not have respiratory or nervous signs of ND. Both groups of TCND-vaccinated birds and one of the groups of CV-vaccinated birds had no decrease in egg production (fig. 5). The second group of CV-vaccinated birds had a sudden decrease in production, beginning 3 days after exposure. Production had stopped by the ninth day, but began a gradual return toward normalcy by the end of the experiment. As shown (table 2), 15 of the 16 birds vaccinated with the TCND vaccine had a rise in titer, indicating spread of infection from contact birds to the vaccinated birds.

Of the birds in two units in which the CV-vaccinated birds were kept, those in unit 1 showed a decided increase in HI titers. Spread of the disease in the group was thus apparent, and the immunity was not sufficient to protect the birds against a decrease and cessation of egg production. In unit 4, in which only 2 of 8 birds had a rise in HI titer, there was no significant decrease in egg production. It is apparent that the birds in unit 4 did not become uniformly infected with the virulent virus and, therefore, did not have a decrease in egg production.

DISCUSSION

Although numerous studies have been made of Newcastle disease, it is apparent that immunity to this disease is composed of many measurable, as well as unmeasurable, factors. This is further complicated by variables introduced by the different strains of virus used in vaccines and by the methods, routes, and doses of virus used for challenge.[1,2,5-9,11] It is obvious that evaluation of immunity in the chicken is not possible until the mechanism of protection in the bird *per se* is better understood.

The use of TCND, which can be administered to chickens without spreading from vaccinated to penmate controls, gave an opportunity to study replacement flocks during their productive lives. Evaluation of immunity in vaccinated chickens by contact exposure to birds affected with the GB strain of NDV showed that two doses of TCND, given eight weeks apart, protected the birds against a decrease in egg production. Except for 1 bird, no signs of ND were observed after contact exposure. A high degree of resistance was substantiated by the maintenance of egg production in the group of birds whose immunity was challenged intramuscularly (fig. 4).

One chicken vaccinated with the TCND vaccine and 2 vaccinated with CV vaccine had râles about eight days after exposure. However, the susceptibility of the respiratory epithelium could not be accurately determined with the methods employed, particularly the relationship between the susceptibility of the respiratory epithelium and protection against decreased egg production. Previous findings, however, with birds in individual cages, showed that respiratory signs were independent of a pause in egg production. It thus appears that resistance to infection of the respiratory epithelium, immunity to systemic infection as denoted by clinical signs, and resistance to a decrease in egg production may be independent of each other The degree and extent of protection of any one of the systems may depend on the dose and the method of challenge or a factor contributing to the degree of immunity which has not been previously evaluated.

A lack of protection offered by the CV vaccine against a pause in egg production was well illustrated in this trial. Although CV-vaccinated birds were well protected against clinical signs of the disease (except 1 bird), decrease in egg production was marked and typical in 86-week-old birds. These results show that the TCND was capable of producing a more complete immunity that was evident to at least 101 weeks of age. The inactivated CV vaccine apparently did not protect the birds against a defect or injury in the physiology or anatomy of the reproductive organs, or perhaps against injury of certain endocrine glands that secrete hormones associated with egg formation.[10]

The irregularity in degree of increase in HI titer was no greater after challenge by contact than after i.m. challenge. However, the susceptible controls (12 in lots 1 and 2) that survived contact challenge showed a minimum increase in HI titer of six doubling dilutions. It is reasonable to assume that the variations in HI response in vaccinated chickens reflect variations in degree of immunity among individuals, not variations in dose or degree of exposure. Since there was no decrease in egg production in TCND-vaccinated birds, a rise in HI titer cannot be used as a sole guide to determine the immunity of the reproductive tract to ND. A resistance to a marked anamnestic

response after exposure may be more indicative of the quality of the immunity.

SUMMARY

Two doses of tissue culture-modified Newcastle disease virus (TCND), administered eight weeks apart to a replacement flock of 28,000 chickens, protected the layers for at least 101 weeks against a decrease in egg production and against signs of the disease after exposure by contact or by intramuscular inoculation of the virulent Texas-GB-1946 strain of Newcastle disease virus.

No spread of TCND between the vaccinated birds and unvaccinated pen contact controls was evident over the period of the experiment.

A flock of 3,500 chickens with signs of delayed reaction to infectious bronchitis vaccination were inoculated with TCND without aggravation of clinical signs.

Chickens vaccinated during the growing stage with two doses of crystal violet-inactivated vaccine and then exposed by contact when 76 weeks of age were protected against signs of Newcastle disease and death, but not against a decrease in egg production.

The systems and relationships involved in a satisfactory and practical immunity to Newcastle disease in laying hens was discussed.

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SUMMARIO IN INTERLINGUA

Immunitate e le Vias Reproductive de Gallinas Ponente, Vaccinate con le Histoculturate Vaccino de Morbo de Newcastle

Duo doses de histoculturalmente modificate virus de morbo de Newcastle, administrate con un intervallo de octo septimanas a un grege de reserva de 28.000 gallinas, protegeva le ponentes durante al minus 101 septimanas contra un reduction del production de ovos e contra signos del morbo post exposition per contacto o per inoculation al virulente racia Texas-GB-1946 de virus del morbo de Newcastle.

Nulle propagation del protection ab le vaccinate aves al non-vaccinate controlos in le mesme gallinario esseva evidente durante le periodo del experimento.

Un grege de 3.500 gallinas con signos de reaction tardive al vaccination de bronchitis infectiose esseva inoculate con le mesme modificate virus de Newcastle, sin ulle aggravation del signos clinic.

Gallinas vaccinate durante le stadio de crescentia con duo doses de vaccino a inactivation per violetto crystallo e exponite subsequentemente, al etate de 76 septimanas, a morbo de Newcastle per contacto esseva protegite contra signos del morbo e contra le morte ab illo sed non contra un reduction de lor production de ovos.

Es discutite le systemas e relationes al base de un satisfacente e practic immunisation contra morbo de Newcastle in gallinas ponente.

Ecologic Considerations in Turkey Ornithosis

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ANY STUDY of an epizootic caused by a member of the psittacosis group of organisms (genus Miyagawanella) offers a rigorous exercise in ecology. Meyer [11] recently reported that at least 98 species of birds, 57 in the parrot family, have been spontaneous hosts to psittacosis organisms. Psittacine birds continue in their classic role as chronically infected carriers of virulent miyagawanellae, which are excreted in large numbers when their host is placed under unfavorable circumstances. Other species of birds and mammals readily become infected when they inhale particles of desiccated excrement of infected birds, and the whole spectrum of disease processes in the new hosts ensues.

There is considerable evidence that certain avian miyagawanellae have found a comfortable ecologic niche in nonpsittacine hosts, such as those of the Columbidae family which includes pigeons and doves.[1, 3, 4, 9] Pigeons are often chronically infected with ornithosis organisms, whether in their natural environment or in captivity, though the infection does not normally progress to clinical disease. The infectious agent in such cases is perpetuated by transfer between parent and nestling. Sporadic excretion of viable miyagawanellae in the nest provides occasional exposure of the squab to the infectious agent. This environment naturally selects those individuals best able to survive ornithosis infection. The nestling matures and continues the cycle. Serologic studies under such conditions indicate that the rate of infection of flock members is often very high, though the disease process, as determined clinically and at necropsy, is symptomless and lesionless unless additional factors upset the host-parasite balance. Concurrent trichomoniasis, salmonellosis, and ornithosis has been observed by the author as the cause of acute disease and fatality in pigeons.[14]

Uncomplicated psittacosis-ornithosis infections are acute and sometimes fatal in susceptible incidental hosts. In the author's opinion, the domestic turkey is such an incidental host. Ornithosis in domestic turkeys has been recognized only within the last ten years and has affected principally breeder flocks in Texas, Oregon, California, Minnesota, and Wisconsin (table 1). Two basic observations that have emerged from studies of ornithosis epizootics in turkeys in those states are: (1) There are gross differences in pathogenicity among the ornithosis agents isolated from turkeys in various states, and (2) the source of the disease agent in any epornitic of ornithosis in turkeys has never been conclusively shown; nevertheless, in two of three diagnosed ornithosis epornitics in California turkeys, ornithosis agents have been recovered simultaneously from other avian species in the immediate vicinity of the affected turkeys.

It is the purpose of this report to present experimental evidence which proves subtle differences in pathogenicity for laboratory animals of ornithosis organisms isolated from California turkeys and which suggests a possible source of ornithosis infection in California turkeys.

MATERIALS AND METHODS

All strains of ornithosis organisms used in this study were isolated by the author from California birds by methods previously described.[12] The C-2 and C-4 strains of ornithosis agents were isolated in 1956 and 1958 from turkeys in California [2, 13] and were serially propagated in mice. A homogenate of infected mouse spleens and livers was used as the source of the infectious agent in studies comparing the pathogenicity of the C-2 and C-4 isolates for six species of birds and mammals. The homogenate was decimally diluted in beef heart broth and 0.1 cc. of each dilution was inoculated on the same day into each of 5 animals of every species by the routes indicated (table 2). Clinical observations were made daily on all animals for two weeks. Those surviving this period were killed and necropsied. Attempts were made to recover

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ornithosis organisms from all affected species; $l.d._{50}$ and $i.d._{50}$ determinations were based on clinical and pathologic observations and were calculated by the method of Reed and Muench.[15]

An ornithosis agent (CP-1 strain) isolated from California pigeons in 1956 was used for the attempts to transmit ornithosis from pigeons to turkeys. There was no known connection between this strain and any avian species other than pigeons prior to the studies on transmission.

All animals used in the experiments were obtained from California commercial sources and were tested prior to experimental use for the presence of ornithosis antibodies in their serums or presence of miyagawanellae in the tissues of hatchmates or penmates. White Cross or Broad Breasted Bronze turkeys and White King pigeons were used for studies of both pathogenicity and experimental transmission of ornithosis.

The complement-fixation-inhibition (CFI) test of Karrer, *et al.*[7] and the capillary-tube agglutination (CTA) test of Mason[8] was used for detecting ornithosis antibodies in the serums of turkeys. A direct CF method of Meyer and Eddie was used to detect ornithosis antibodies in the serums of pigeons and guinea pigs.

RESULTS

Pathogenicity Studies.—The differences in pathogenicity of ornithosis agents isolated from turkeys in Texas and California are reflected in the severity of lesions and mortality in affected birds, in the incidence of related disease in persons handling the birds, and in laboratory studies where the ability of an isolate to produce toxin and to infect and kill laboratory animals can be measured and correlated. Highly pathogenic strains are usually good toxin producers and are referred to as being toxigenic. The toxigenicity of a strain isolated from a turkey in Texas has been characterized by Meyer and Eddie[10] who showed that the toxin produced by this strain was antigenically distinct from toxins produced by certain other avian and mammalian strains. Antigenic specificity of toxins produced by different strains of psittacosis-ornithosis-pneumonitis organisms has, therefore, been used as the basis for speciation within the genus Miyagawanella.

Agents isolated from California turkeys were readily distinguished from strains isolated from turkeys in Texas and Oregon by toxin titrations or infectivity titrations in mice.[6,10,12] Even the less toxigenic strains isolated from California turkeys differed from each other markedly in their effects on laboratory animals. As shown (table 2), the C-2 and C-4 strains of ornithosis agents differed greatly in their infectivity and lethality for pigeons and turkeys but were not significantly different in their pathogenicity for chicken embryos, mice, guinea pigs, or parakeets. The differences in their effects on turkeys and pigeons were revealed only by the titration technique; for when the effects of equal numbers of organisms of the C-2 and C-4 strains on individual laboratory species were compared (using pigeon intracerebral $i.d._{50}$ as the basis for determining the absolute numbers of infectious particles present in the

TABLE 1—Reported Incidence of Ornithosis in Turkeys and Related Human Cases in the United States and Canada 1948-1958

Year	State	No. locations	Isolation of ornithosis agent from turkeys	No. human cases of psittacosis related to infected turkeys
1948	Texas	1	No	22
1951–52	Texas	3	Yes	75
1954	Texas	6	Yes	200
	New Jersey	1	Yes (NJ-1 strain)	17
	California	1	Yes (C-1 strain)	None known
	Wisconsin-Minnesota	3	Yes	None known
	Iowa	1?	No	3?
	Michigan	1	Yes	None known
1956	Texas	2	Yes	30
	Oregon	2	Yes	86
	California	1	Yes (C-2 strain)	None known
	Wisconsin	1	Yes	9
	Washington	1?	No	4?
1957	British Columbia	1	Yes	27
	Oregon	1	Yes	1
	Minnesota	2	Yes	None known
1958	California	1	Yes (C-4 strain)	None known
	Oregon	1	Yes	None known

original suspensions), it was apparent that the C-2 strain was at least 30 times more infective for turkeys than the C-4 isolate. Moreover, the C-4 strain was over 525 times more lethal than the C-2 strain for pigeons inoculated by the intracerebral route. Therefore, there were differences in pathogenicity between two strains of ornithosis agents which appeared identical when inoculated into mice or chicken embryos.

TABLE 2—Results of Titration of Broth Suspensions of Two Turkey Ornithosis Agents in Six Species of Birds and Mammals

Species	Age	Route of inoculation	Suspension of C-2 isolate		Suspension of C-4 isolate	
			$l.d._{50}$/ml.	$i.d._{50}$/ml.	$l.d._{50}$/ml.	$i.d._{50}$/ml.
Chicken embryo	7 days	Yolk sac	68,000	.....	100,000	.....
Turkey	12 weeks	Intraperitoneal	0	10,000	0	1,000
Pigeon	Adult	Intracerebral	56	320,000	230,000	1,000,000
Parakeet	Adult	Intraperitoneal	68	100,000	(50)*	(100,000)*
Mouse	4 weeks	Intraperitoneal	0	43,000	0	68,000
Guinea pig	8 weeks	Intraperitoneal	0	0	0	0

* An estimate based on an incomplete titration.

Transmission Studies.—In 1956, the tissues of 3 of 15 pigeons, that had been roosting on a turkey ranch for one year prior to an ornithosis outbreak in the turkeys, contained ornithosis agents morphologically and antigenically indistinguishable from an agent isolated from sick turkeys on the same premises.[2] In this case, the pigeons carried the disease agent without visible disease. In 1958, sparrows nesting near ornithosis-infected turkeys were found to carry, also without obvious disease, an ornithosis agent similar to the one isolated from the diseased turkeys.[13] On the basis of the 1956 experience, an effort was made to determine experimentally whether normal turkeys could actually be infected by being placed in an enclosure containing ornithosis-infected pigeons. Therefore, 5 normal mature pigeons were inoculated intratracheally and intraperitoneally with 2,000 mouse $i.d._{50}$ of an ornithosis agent of pigeon origin. The pigeons were placed in a wire cage hanging from the ceiling of an enclosure containing 10 normal turkeys, 17 weeks of age. The birds were observed daily, and blood was taken biweekly from both species for serologic testing by the CFI and CTA tests.

TABLE 3—Experimental Transmission of Pigeon Ornithosis Agents from Pigeons to Turkeys by Pen Contact: Serologic Results

Group	Serologic test	No. birds with positive serologic response, Week of test								
		0	2	4	6	8	10	12	14	16
5 Pigeons* (inoculated with 2,000 mouse $i.d._{50}$ pigeon ornithosis agent)	DCF	0	1	1	1	1	..	..	..	..
10 Turkeys (pen contact with above pigeons)	CFI	0	2	6	9	8	7	7	4	2
	CTA	0	0	0	0	7	7	9	8	4
5 Turkeys (pen contact with above turkeys at 8th week)	CFI	..	..	..	..	0	0	2	2	1
	CTA	..	..	..	..	0	5	5	4	2
4 Turkeys (pen contact with above turkeys at 13th week)	CFI	..	..	..	..	..	..	0	0	1
	CTA	..	..	..	..	..	..	0	2	1

* Pigeons were removed at eighth week of test.

By the second week of the test, a serologic response was observed in 2 of the turkeys and 1 of the pigeons, and within six weeks, 9 of 10 turkeys developed CFI titers ranging from 1:4 to 1:32, thereby estab-

lishing that infection of the birds had occurred (table 3).

The study was continued to determine whether the ornithosis agent picked up by the turkeys from the pigeons could be transmitted in turn to a new group of normal susceptible turkeys. The pigeons were removed at the eighth week of the test, and 5 unexposed turkeys, along with the turkeys originally infected by an agent from the pigeons, were placed in clean quarters. The second group of turkeys in turn developed serum antibody titers within two weeks. A third group of 4 birds was placed in the pen with the other infected birds on the thirteenth week of the test. Again, 3 of these birds responded serologically within three weeks.

At the sixteenth week, the test was terminated, and the remaining birds were killed. No distinct lesions of ornithosis were observed in any of the birds, nor were signs of disease visible in any of the birds during the entire test. The turkeys appeared to have undergone a subclinical, transient ornithosis infection. Most birds experienced a rise and fall in serum antibody titer. Attempts to isolate the infectious agent at various intervals during the test and at termination failed.

This experiment was replicated with the exception that in the second test turkeys were inoculated intraperitoneally with the pigeon ornithosis agent instead of being exposed to ornithosis-infected pigeons. Virtually identical serologic results were obtained in both tests.

Discussion

In considering the source of the disease agent in ornithosis epornitics in turkeys, one must first ask: Is the domestic turkey now a natural reservoir of ornithosis organisms? It is conceivable that turkeys are a potential reservoir, but mechanical incubation of turkey eggs, artificial brooding of poults, and slaughter of breeders obviates the chance of perpetuating the disease agent through generations of turkeys in a manner similar to that of psittacine and columban species.

Contamination of a turkey-raising area with ornithosis agents excreted by an infected flock offers a good opportunity for transmission of an agent through generations of turkys, but this is merely a means of transmission, without significant bearing on the question of the source of the disease agent on previously uncontaminated premises.

If transovarian passage is excluded as a means of perpetuating ornithosis agents in turkeys (and all field and experimental evidence excludes it thus far [5, 13]), then one must assume that the turkey is essentially an incidental host to these agents, and that each new epornitic is initiated by miyagawanellae excreted by other species intermingling with the turkey. While avian species are the most likely species to be labeled as the intermingling carrier, rigorous ecologic investigations must exclude nonavian hosts.

In the aforementioned cases of close association of several affected avian species, such as pigeons and turkeys, it is difficult to ascertain which species transmitted the agent to the other, or whether a third species was involved as the original carrier or intermediate host. The finding of healthy, avian carrier hosts associated with two ornithosis epornitics in California turkeys nevertheless is suggestive of the role of free-flying birds in the transmission of ornithosis to turkeys. Moreover, the experimental evidence showing that two strains of ornithosis agents (isolated from turkeys on ranches where other avian species were shown to carry similar ornithosis agents) differed pathogenically suggests that the source hosts in each epornitic were different. The experiment showing transmission of an agent from pigeons to turkeys confirms the possibility of the natural occurrence of inter-avian transfer of miyagawanellae.

Examination of the serologic results in the transmission experiment proved highly interesting. Previous experience had shown that agglutinins detectable by the CTA test had invariably appeared prior to CFI antibodies in the serums of turkeys undergoing infection with an ornithosis agent of turkey origin.[14] Yet, that order was reversed in the first group of turkeys exposed to infected pigeons. Positive CFI titers had preceded by eight weeks the appearance of CTA agglutinins in the serums of 9 of the 10 birds exposed to infected pigeons.

In contrast, alternate susceptible turkeys, newly placed in contact with infected tur-

keys on the eighth and thirteenth weeks of the test, developed serum agglutinins within two weeks, with the appearance of CFI antibodies trailing by a week. These serologic reactions represented a return to the normal course of antibody development when turkeys were infected with agents of turkey origin.

The significance of this reversed serologic reaction is better understood when one becomes cognizant of the character of the antigens used in the CFI and CTA tests. The former test employs an antigen prepared from yolk-sac propagated psittacosis organisms which have been boiled and phenolized. This process destroys all specific surface antigens but exposes the common group antigen found in all members of the psittacosis-LGV group. The CTA-antigen, on the other hand, is made up of whole elementary bodies of the NJ-1 turkey ornithosis agent which have been mechanically purified and stained with Giemsa's solution. Normally, turkeys infected with known turkey ornithosis agents develop serum agglutinins detected by the CTA antigen prior to antibodies detected by the CFI test.

The reversed serologic effect suggested that the antigenic constitution of the CP-1 pigeon ornithosis agent may have changed during its growth in turkeys, and that this changed antigenic structure was more similar to the NJ-1 turkey ornithosis agent than before, thereby influencing the production, during the eight weeks, of antibodies detected by the CTA test. This theory is speculative, but an antigenic change of this type could signify the initial step in the evolution of a turkey ornithosis agent.

The natural and experimental evidence mentioned here, though fragmentary and incomplete, emphasizes the need not only for ecologic investigations ancillary to ornithosis epizootiology but also for critical experimentation that sharply defines the differences and similarities between strains of organisms isolated from domestic turkeys and other birds within the turkey environment. Laboratory investigations will require new approaches beyond the basic characterization of each new isolate for its pathogenicity, antigenicity, and immunogenicity. The genetics and biochemistry of the production of the toxin found associated with these organisms deserves careful review and further study. Production of toxin is one of the keys to the pathogenicity of ornithosis organisms; therefore, an explanation of the mechanism of its elaboration has a direct bearing on the appearance of toxigenic miyagawanellae in birds.

SUMMARY

Two strains of ornithosis agents isolated from turkeys in California were found to differ markedly in their pathogenicity for turkeys and pigeons.

Replicate experiments on the transmission of ornithosis showed that pigeon ornithosis agents could be transmitted by natural means from pigeons to turkeys and that the infectious agent multiplied in the turkey host sufficiently to stimulate antibody production. The agent was excreted by the turkeys and was transmitted in turn to alternate susceptible turkeys placed in pen contact with turkeys originally exposed to infected pigeons.

The significance of these two experiments in relation to the ecology and epizootiology of ornithosis in turkeys and other birds is discussed.

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SUMMARIO IN INTERLINGUA

Considerationes Ecologic Relative a Ornithosis del Gallo de India

Esseva constatate que duo racias del agente de ornithosis, isolate ab gallos de India in California, differeva marcatemente in lor pathogenicitate pro gallos de India e pro columbas.

Experimentos repetitori concernente le transmission de ornithosis monstrava que le agente de ornithosis de columba poteva esser transmittite per medios natural ab columbas a gallos de India e que le agente infectiose se multiplicava in le gallos de India sufficientemente pro stimular le production de anticorpore. Le agente esseva excernite per le gallos de India e transmittite a altere susceptibile gallos de India placiate in contacto con le gallos originalmente exponite a inficite columbas.

Le signification de iste duo experimentos con respecto al ecologia e al epizootiologia de ornithosis in gallos de India e altere aves es discutite.

Teniacidal Trials with Some Diphenyl Sulfones in Cats, Dogs, and Chickens

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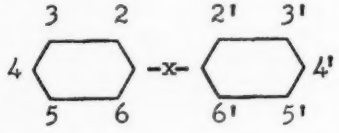
CERTAIN substituted bis-phenols have exhibited teniacidal action in domestic animals, poultry, and man. Dichlorophen [bis-(2-hydroxy-5-chlorophenyl) methane] has been reported to be effective against *Taenia pisiformis* and *Dipylidium caninum* in dogs,[2] Taenia spp. in cats,[1] *Thysanosoma actinioides* and Moniezia spp. in sheep,[10] *Choanotaenia infundibulum* and *Raillietina cesticillus* in chickens,[4] and *Taenia saginata* and *D. caninum* in man.[6] Hexachlorophene [bis(2-hydroxy-3,5,6-trichlorophenyl)methane] has exhibited marked teniacidal action against *R. cesticillus* in chickens.[7] Several other diphenyl methanes, a diphenyl propane [bis(3-hydroxy-2,4,5,6-tetrachlorophenyl) propane], and a diphenyl ether [bis(2-hydroxy-3,4,5,6-tetrachlorophenyl) ether] have shown limited action against *R. cesticillus* in chickens.[8]

These chemicals have the following general structure:



The X-group is either $-CH_2-$(methane), $-CH_2 \cdot CH_2 \cdot CH_2-$(propane), or -O- (ether). A number of substituted diphenyl sulfones,* chemicals of this type in which the X-group is an $-SO_2-$ radical, became available for testing in 1950. Anthelmintic trials with these substances seemed desirable because of their structural relationship with the chemicals previously noted and because antiparasitic activity had been reported for other sulfones.[3,11] The trials were conducted at irregular intervals over a period of about eight years (1950 to 1957) as circumstances allowed and as suitable test animals became available. Preliminary data were published [12] in 1951.

From the Animal Disease and Parasite Research Division, Agricultural Research Center, USDA, Beltsville, Md.

* Supplied by Monsanto Chemical Co., Organic Chemicals Division, St. Louis, Mo.

MATERIALS AND METHODS

The dogs and cats were of mixed breeds and ranged in age from 4 months to about 7 years. Most of them had natural infections of Taenia spp., and 1 cat harbored *D. caninum*. Experimental infections with *T. pisiformis* and *Taenia taeniaformis* were established in a few of the animals. Several tapeworm-free cats were included in various dosage groups to provide additional information on tolerance of the chemicals. The chickens were Rhode Island Red and Plymouth Rock cockerels and pullets about 3 or 4 months of age; they had been experimentally infected with *R. cesticillus*.

Most of the dogs also harbored one or more species of intestinal nematodes commonly found in this area, namely, *Toxocara canis, Toxascaris leonina, Ancylostoma caninum,* and *Trichuris vulpis. Toxocara cati* was frequently encountered in the cats, and *A. caninum* and Physaloptera spp. occurred occasionally. None of the chemicals exhibited significant action against nematodes.

The testing procedures in dogs and cats were essentially as described previously [5] and, in chickens, a modification of the controlled test described by Moskey and Harwood [9] was followed.

RESULTS

The data obtained in trials with cats and dogs are summarized (tables 1, 2). Bis(3-chloro-4-hydroxyphenyl)sulfone was completely effective against 17 *T. taeniaformis* in 4 cats that were given a dosage of 100 mg./lb. of body weight. At the slightly lower dose rate of 75 mg./lb., however, the chemical removed only 7 (28%) of 25 Taenia from 8 cats and was totally ineffective against 17 Dipylidium in 1. Four of 19 cats vomited, and a softening of the feces occurred in 1.

Bis(2-hydroxy-5-chlorophenyl)sulfone, at dose rates ranging from 40 to 100 mg./lb. of body weight, removed 65 (93%) of 70 Taenia from 13 cats; 4 of the 5 tapeworms recovered at necropsy were obtained from 1 animal that was given the lowest dosage. The chemical also showed marked teniacidal action in dosages of 25 and 30 mg./lb., removing, in the aggregate, 191 (91%) of 209 Taenia from 31 animals. In lesser amounts, the efficacy was variable and unreliable. The lowest dosage employed in these trials,

TABLE 1—Teniacidal Action of Some Sulfones in Cats

Sulfone	No. of animals	Dosage (mg./lb.)	Parasites	Removed	Left	Efficacy (%)	Complete clearance*	Remarks
Bis(3-amino-4-hydroxyphenyl)	1	100	Taenia	0	4	0	0/1	.....
Bis(3-nitro-4-chlorophenyl)	1	100	Taenia	0	2	0	0/1	.....
Bis(2-hydroxy-3-chlorophenyl)	3	100	Taenia	2	13	13	1/2	.....
	5	100	Taenia	17	0	100	4/4	3 vomited
Bis(3-chloro-4-hydroxyphenyl)	9	75.0	Taenia	7	18	28	2/8	.....
			Dipylidium	0	17	0	0/1	.....
	5	50.0	Taenia	3	19	13	1/4	1 vomited; soft feces in 1
	7	100	Taenia	37	1	97	3/4	5 vomited
	6	50.0	Taenia	22	0	100	4/4	.....
	7	40.0	Taenia	6	4	60	4/5	1 vomited; soft feces in 4
	12	30.0	Taenia	68	10	87	8/11	1 vomited; soft feces in 2
Bis(2-hydroxy-5-chlorophenyl)	25	25.0	Taenia	123	8	93	14/20	1 vomited; soft feces in 5
	9	20.0	Taenia	15	28	35	2/8	.....
	8	15.0	Taenia	12	16	42	2/4	.....
	6	12.5	Taenia	1	19	5	1/4	.....
	5	10.0	Taenia	63	18	77	1/4	.....

* Number cleared/number infected.

10 mg./lb. of body weight, removed an aggregate 77 per cent of 81 Taenia from 4 cats. The disproportionately high efficacy at this dose rate, however, was ascribable largely to 1 animal which eliminated all of 53 tapeworms. Complete removal of tapeworms occurred in at least 1 animal at each dosage level. Eight of 85 cats vomited, and 11 passed soft feces.

Bis(3-amino-4-hydroxyphenyl) sulfone, bis(3-nitro-4-chlorophenyl)sulfone, and bis-(2-hydroxy-3-chlorophenyl)sulfone showed no promise of effective action when given at the rate of 100 mg./lb. of body weight.

In critical trials with dogs, only bis(2-hydroxy-5-chlorophenyl)sulfone exhibited significant teniacidal action. The chemical was completely effective against 3 Taenia in 2 dogs that were given a dosage of 100 mg./lb. of body weight, and at 50 mg./lb., the chemical removed all of 5 Taenia from 1 dog but none of 63 from another. After treatment, all 4 dogs either vomited or passed soft feces or both. In a privately owned dog, which was not available for necropsy, a dosage of 100 mg./lb. of body weight was apparently effective on one occasion but ineffective at a later date following reinfestation.

Bis(dichloromethyl)sulfone,* bis(3-amino-4-hydroxyphenyl)sulfone, bis(3-nitro-4-chlorophenyl)sulfone, bis(2-hydroxy-3-chlorophenyl)sulfone, and bis(3-chloro-4-hydroxyphenyl)sulfone were uniformly ineffective against Taenia in limited trials at dose rates of 50 and 100 mg./lb. of body weight.

In preliminary trials with chickens, bis-(3-chloro-4-hydroxyphenyl)sulfone was ineffective against 57 *R. cesticillus* in 1 of 2 birds that were given a dosage of 200 mg./kg. of body weight; 1 bird was not infected with tapeworms. Bis(2-hydroxy-5-chlorophenyl)sulfone, given to 2 birds at a dose rate of 200 mg./kg., was ineffective against 97 Raillietina; at 400 mg./kg., the chemical showed no promise of effective action against 8 Raillietina in 2 birds.

DISCUSSION

Bis(2-hydroxy-5-chlorophenyl)sulfone exhibited marked teniacidal action against *T. taeniaformis* in cats and showed promise of effective action against *T. pisiformis* in dogs. It was ineffective against *R. cesticillus*, however, in limited trials with chickens.

* Although this chemical does not have the diphenyl structure of other members of the series, it was included because of its chemical affinities in other respects.

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The chemical was reasonably well tolerated in effective dosages. Emesis and soft feces, the former unassociated with symptoms of systemic disturbances, were the only indications of toxicity. Cats appeared to be more tolerant of the chemical than dogs, although comparatively few trials were made with the latter host.

The optimum dosage for cats was not established definitively, but the data suggest that it probably approximates 25 to 50 mg./lb. of body weight. In this dosage range, the chemical removed 90 per cent of 242 Taenia from 40 cats and achieved complete clearance in 30 of the animals. Soft feces were passed by 11 of 50 cats but only 3 vomited, attesting the comparative safety of the chemical for these animals. The data in dogs are too limited to permit a reasonable determination of optimum dosage.

Among the other compounds employed in these trials, only bis(3-chloro-4-hydroxyphenyl)sulfone exhibited significant teniacidal action in cats. The highest dosage, 100 mg./lb. of body weight, was completely effective against Taenia, but 3 of 5 cats vomited, suggesting that this dosage probably approximates the maximum feasible limit. It is noteworthy also that the chemical showed no action against Dipylidium at a slightly lower dose rate.

Although the data are limited and do not cover a sufficiently broad group of compounds to permit significant comparisons, the apparent indications are of some interest. The kind and spatial arrangement of substituents in the sulfone nucleus seemed to have a bearing on anthelmintic activity in cats. Hydroxyl and chlorine substituents were both present in compounds that showed teniacidal action, and those compounds in which either was replaced by amino or nitro groups were totally ineffective. Bis(3-chloro-4-hydroxyphenyl)sulfone, at a dose rate of 100 mg./lb. of body weight, was wholly effective against Taenia; but substitution of amino groups for the chlorine atoms resulted in a compound [bis-(3-amino-4-hydroxyphenyl)sulfone] completely devoid of teniacidal action at a similar dose rate. The lack of action was not ascribable solely to the absence of the chlorine atoms, however, because substitution of nitro groups for the hydroxyl radicals [bis(3-nitro-4-chlorophenyl)sulfone] did not restore anthelmintic activity. This may indicate that both hydroxyl and chlorine substituents are essential, although the possible effect of the change in position of the chlorine atoms in this instance cannot be discounted.

The influence of spatial arrangement on teniacidal action is suggested by the results obtained with bis(2-hydroxy-3-chloro-

TABLE 2—Anthelmintic Action of Some Sulfones Against Taenia pisiformis in Dogs

Sulfone	Dosage (mg./lb.)	Dog. No.	Parasites		Efficacy (%)	Remarks
			Removed	Left		
Bis(dichloromethyl)	50.0	573	0	+(b)	0	Incoordination
		673	0	47	0	Incoordination
	100	674	0	6	0	Incoordination
Bis(3-amino-4 hydroxyphenyl)	50.0	560	0	12	0	.....
	100	668	0	2	0	Soft feces
Bis(3-nitro-4-chlorophenyl)	50.0	671	1	20	4	.....
	100	672	0	16	0	.....
Bis(2-hydroxy-3-chlorophenyl)	50.0	574	0	1	0	.....
	100	669	0	9	0	Soft feces
Bis(3-chloro-4-hydroxyphenyl)	50.0	675	0	4	0	.....
	100	513	0	11	0	.....
Bis(2-hydroxy-5-chlorophenyl)	12.5	540(a)	0	63(c)	0	Soft feces
	25.0	541	1	6	14	.....
	50.0	542	5	0	100	Vomited; soft feces
		540	0	63	0	Vomited
	100	512	1	0	100	Soft feces
		674	2	0	100	Vomited; soft feces

(a)= Not necropsied; treated subsequently at 50 mg./lb.; (b)= not necropsied; (c)= determined after subsequent test.

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phenyl)sulfone and its 4-hydroxy and 5-chloro isomers. Changing the hydroxyl radicals from the ortho to the para positions [bis(3-chloro-4-hydroxyphenyl)sulfone] resulted in a substantial increase in teniacidal activity and moving the chlorine substituents to the 5- positions produced an even greater increase in anthelmintic action.

It is noteworthy that the efficiency of bis(2-hydroxy-5-chlorophenyl)sulfone compares favorably with that of its methane analogue, dichlorophen, against Taenia spp. in cats and dogs. On this account, it seemed especially desirable to investigate the potential teniacidal action of the sulfone analogue of hexachlorophene, but experimental quantities of this and other desired halogenated hydroxyphenyl sulfones have not been available.

SUMMARY

In critical trials with several substituted diphenyl sulfones, one, bis(2-hydroxy-5-chlorophenyl)sulfone, exhibited marked anthelmintic action against *Taenia taeniaformis* in cats and, to a lesser extent, *Taenia pisiformis* in dogs. It showed no promise of effective action against *Raillietina cesticillus* in limited trials with chickens.

The chemical was well tolerated in effective dosages, especially in cats. Emesis and soft feces, the only signs of toxicity, occurred infrequently at dose rates approximating the optimum range, *i.e.*, 25 to 50 mg./lb. of body weight.

The relationship of chemical structure and anthelmintic action is discussed briefly.

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SUMMARIO IN INTERLINGUA

Essayos Teniacida con Certe Sulfones Diphenylic in Cattos, Canes, e Gallinas

In essayos critic con plure substituite sulfones diphenylic, un de illos—bis(2-hydroxy-5-chlorophenyl)-sulfona—exhibiva un marcate activitate antihelmintic contra *Taenia taeniaformis* in cattos e, minus extensemente, contra *Taenia pisiformis* in canes. Illo non promitteva un efficace activitate contra *Raillietina cesticillus* in restringite essayos con gallinas.

Le composito esseva ben tolerate in doses efficace, specialmente per cattos. Emesis e feces dulce—le sol signos de toxicitate—occurreva infrequentemente a dosages vicin al optimo, *i.e.*, inter 25 e 50 mg per libra de peso corporee.

Le relation inter le structura chimic e le action antihelmintic es discutite brevemente.

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Preliminary Trials with Bithionol Against Tapeworm Infections in Cats, Dogs, Sheep, and Chickens

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THE INJURY produced by tapeworms in dogs and cats has not been clearly defined. The control of these parasites is important, however, because of the transmissibility of certain stages to man and livestock and because of the nuisance associated with them in pet animals. The clinical significance of adult tapeworm infections in food-producing animals is also conjectural, although their economic importance and pathogenic potential under conditions of stress are generally acknowledged. By and large, the chemical agents currently used against these parasites in domestic animals and poultry are less reliable and are potentially more toxic than those employed against other helminthic species. The need for safer and more uniformly effective remedies, therefore, is evident.

In preliminary antiparasitic investigations with miscellaneous chemicals, bithionol,* exhibited teniacidal action in dogs, cats, sheep, and chickens. The results of these trials, most of which were conducted in 1952 and 1953, are summarized at this time despite their limited scope because Sawada [4,5] recently reported his findings on the action of the chemical against Raillietina infections in chickens, and because it is unlikely that we shall resume these investigations in the immediate future.

Bithionol is a white crystalline solid with a faint phenolic odor. Its water solubility and specific gravity at 25 C. are 0.004 per cent and 1.61 ± 0.01, respectively. The chemical has marked bacteriostatic activity and is effective also against certain pathogenic fungi. Its structural formula is shown (fig. 1).

EXPERIMENTAL PROCEDURE

The dogs and cats used in this experiment were of mixed breeds and ranged in age from about 3 months to 6 years. Most of them harbored naturally acquired infections of Taenia spp., and 1 dog had a concomitant infection of Dipylidium spp. A few tapeworm-free animals were included to provide additional information on the tolerance of the chemical. The lamb had a naturally acquired infection of *Moniezia expansa*. The chickens had experimental infections of *Raillietina cesticillus;* each bird had been given 25 cysticercoids obtained by dissection from meal beetles (Tribolium spp.).

The testing procedures varied slightly with the different host-parasite systems. In dogs, cats, and sheep, they were essentially as described heretofore;[1,2] in chickens, the procedure followed the pattern of the controlled test for cestodes described by Moskey and Harwood.[3]

Most of the mammalian hosts harbored one or more species of gastrointestinal nematodes commonly found in this area, but the chemical did not exhibit significant action against any of them.

RESULTS

The data are given in table 1. All of 147 Taenia were removed from 10 cats that were given dosages ranging from 100 to 250 mg./lb. of body weight; at 75 mg./lb., the chemical removed 23 (96%) of 24 tapeworms from 4 cats. The lowest dosage, 50 mg./lb., removed only 9 (42%) of 21 Taenia from 3 cats. The chemical was reasonably well tolerated in all trials, although a moderate to marked softening of the feces frequently occurred with the larger dosages. This reaction was noted, however, in only 4 of 16 animals that were given 75 to 100 mg./lb. of body weight, which seemed to be dosages that approximated the optimal level.

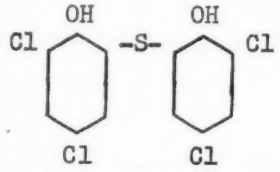

Fig. 1—Structural formula of bithionol.

Bithionol removed all of 50 Taenia from 3 dogs that were given dosages ranging

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* Bithionol [2,2'-thiobis (4,6-dichlorophenol)] was supplied by Monsanto Chemical Co., Organic Chemicals Division, St. Louis, Mo., under the trade name Actamer.

from 100 to 250 mg./lb. of body weight; but at 75 and 50 mg./lb., the aggregate efficacy decreased to 76 and 53 per cent, respectively. The lowest dosage, moreover, was wholly ineffective against 27 Dipylidium in 1 dog. Seven of the 15 dogs vomited, and a moderate to marked softening of the feces was noted in 10.

The chemical was completely effective against Moniezia in 1 lamb, weighing 42 lb., that was given a dosage of 100 mg./lb. of body weight. Part of the feces collected during the first 24 hours after medication were soft, but there were no other unfavorable reactions. The animal gained 5 lb. during the two-week test period.

All Raillietina were removed from 2 chickens that were given 500 mg./kg. of live weight in single doses; 38 tapeworms were recovered from 2 control birds. Lower dosages were correspondingly less effective. In divided doses of 100 mg./kg. daily for five days, bithionol removed about half of the Raillietina from 2 birds; 11 tapeworms were recovered at necropsy from the principals and 21 from 2 controls. There was a softening of the droppings of all birds that were given the chemical in single doses.

DISCUSSION

Bithionol exhibited teniacidal action against Taenia spp. in dogs and cats, *M. expansa* in sheep, and *R. cesticillus* in chickens. The chemical was wholly ineffective, however, against Dipylidium spp. in 1 dog and showed no promise of effective action against any of the common gastrointestinal nematodes in these hosts.

The data are too limted to permit a definitive determination of optimum dosage. They suggest, however, that approximately 100 mg./lb. of body weight would be appropriate for further testing, at least in the mammalian hosts employed in these trials.

Emesis in dogs and a softening of the feces in dogs, cats, chickens, and sheep were the only indications of toxicity. Neither reaction is especially alarming, particularly when unaccompanied by nausea, depression, and subsequent inappetence. Emesis may have an important bearing on efficacy, however, if a substantial part of the dose is ejected with the vomitus.

Curiously, cats seemed to be more tolerant of the chemical than dogs, despite the commonly accepted view that cats are particularly susceptible to intoxication from phenolic compounds. Bithionol was given to both hosts in equivalent dosages; yet none of the cats vomited and alteration in the character of the feces was of a lower incidence and lesser magnitude.

The trials in sheep and chickens are merely suggestive of potential teniacidal action in these hosts. Our findings with chickens were somewhat less promising

TABLE 1—Anthelmintic Action of Bithionol Against Tapeworms

Host	No. of animals	Dosage (mg./lb.)	Parasites	Removed	Left	Efficacy (%)	No. of tests	Remarks
Cat	2	250	Taenia	13	0	100	1	Soft feces in 1
	1	200	Taenia	22	0	100	1	Fluid feces
	2	150	Taenia	34	0	100	2	Soft feces
	8	100	Taenia	78	0	100	6	Soft feces in 2
	8	75	Taenia	23	1	96	4	Soft feces in 2
	5	50	Taenia	9	12	42	3	.....
Dog	1	250	Taenia	24	0	100	1	Emesis; soft feces
	1	200	Taenia	....	..	.....	..	Emesis
	1	150	Taenia	9	0	100	1	Fluid feces
	3	100	Taenia	17	0	100	1	Emesis in 2; soft feces in 3
	6	75	Taenia	52	16	76	6	Emesis in 3; soft feces in 4
	3	50	Taenia	7	6	53	3	Soft feces in 1
			Dipylidium	0	27	0	1	
Sheep	1	100	Moniezia	19*	0	100	1	Soft feces
Chicken	2	500(a)	Raillietina	+	0	100	2	Fluid feces
	2	Controls	Raillietina	0	38	.....	..	.....
	2	400(a)	Raillietina	+	1	87	2	Fluid feces
	2	200(a)	Raillietina	0	14	0	2	Fasted 18 hr.; fluid feces
	1	Control	Raillietina	0	4	.....	..	.....
	2	100(a)(b)	Raillietina	+	11	47	2	.....
	2	Controls	Raillietina	0	21	.....	..	.....

(a) mg./kg.; (b) daily for five days; * cc. water displacement; + strobilae eliminated after treatment.

than those obtained by Sawada.[4,5] He reported "almost complete removal" of Raillietina with a dosage of 100 mg./kg. given twice at an interval of 18 days. We found that this dosage was ineffective when given for five consecutive days.

SUMMARY

In limited trials, bithionol [2,2'-thiobis-(4,6-dichlorophenol)] exhibited teniacidal action in cats, dogs, sheep, and chickens. The optimum dosage was not established, although a dose rate of 100 mg./lb. of body weight was reasonably well tolerated and completely effective against Taenia spp. in dogs and cats and *Moniezia expansa* in sheep. A dosage of 500 mg./kg. of body weight removed all *Raillietina cesticillus* from 2 chickens.

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SUMMARIO IN INTERLINGUA

Essayos Preliminari in le Uso de Bithionol Contra Infectiones Tenial in Cattos, Canes, Oves, e Gallinas

In pauco extense essayos, bithionol—i.e. 2,2'-thiobis-(4,6-dichlorophenol)—manifestava virtute teniacida in cattos, canes, oves, e gallinas. Le dosage optimal non esseva determinate. Tamen, doses de 100 mg per libra de peso corporee esseva satis ben tolerate e se provava completemente efficace contra species tenial in canes e cattos e contra *Moniezia expansa* in oves. Un dosage de 500 mg per kg de peso corporee eliminava omne tracia de *Raillietina cesticillus* in 2 gallinas.

XUM

Incidence and Treatment of Parasites in a Zoological Garden

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IT WAS OBSERVED (Grzimek [2]) that while the human species is rapidly increasing in numbers, the decrease in numbers of many animals has led to extinction in some cases. Extinction in other cases is threatened by wanton slaughter of wild animals. Inroads of agriculture and industry into areas previously inhabited only by animals indicates a greater necessity for care and reproduction of animals in captivity. Binkley [1] pointed out that the use of therapeutic agents (e.g., new anthelmintics) is approached with caution in treating captive animals, regardless of the efficacy of the therapy in man and domestic animals.

Helminthologic investigations of animals in Swiss Zoological Gardens were recorded by Kries.[6] Porter [7,8] reported on the parasites collected from animals in the London Zoological Gardens. Jaskoski and Williamson [4] found an incidence of 53.7 per cent nematode infections in 626 animals at the Chicago Zoological Park. An incidence of 20.8 per cent was reported by Jaskoski [5] for 259 animals at the Lincoln Park Zoological Gardens, Chicago.

An excellent bibliographical index of diseases of wild animals and birds was compiled by Halloran.[3] More recently, Ruch [9] has written a text on diseases of laboratory primates.

This summary brings together information on the occurrence and distribution of parasites in animals of the Lincoln Park Zoological Gardens and Indian Boundary Zoo in Chicago. The survey began in September, 1958, and was concluded in June, 1959. The host-parasite lists included in this report are incomplete and will remain so due to circumstances beyond control, *i.e.*, hibernation of animals, inaccessible excrements, and composites of mixed animals that invalidate the results of investigation.

TABLE 1—Incidence of Parasitic Infection in Zoo Animals

Parasites	No. of animals infected	Percentage of infection
Armillifer armillatus	1	1.92
Ascaris transuga	4	7.69
Coccidia	1	1.92
Parascaris equorum	2	3.84
Strongyloides	8	15.38
Toxocara	13	25.0
Trichuris	10	19.25
Unidentified	11	20.0
Welcomia evoluta	2	3.84

MATERIALS AND METHODS

The animal census of the Lincoln Park Zoological Gardens and Indian Boundary Zoo in Chicago is 2,222 animals, including 4 invertebrates, 90 amphibians, 629 reptiles, 1,124 birds, and 375 mammals. Observations were made of 584 fecal specimens from 194 cages and enclosures which were made available for this survey. Fecal samples were collected in uncontaminated paper cups properly capped and labeled. The specimens were collected before 4:00 p.m. and examined for parasite eggs, larvae, and adults daily. Necropsies were performed on animals which died during the period of this survey to determine if there were any evidences of parasitism.

Sugar flotation techniques with centrifugation were employed for concentration of eggs and larvae in fecal samples. The number of eggs per 4 Gm. of feces was determined by a modification of the Stoll technique for determination of eggs per gram of feces.

ANTHELMINTICS

Hexylresorcinol was the first anthelmintic administered that showed positive parasitic results in this survey. A coated preparation of hexylresorcinol was used to prolong action of the drug by retarding digestive enzyme action so that the drug could be digested in the lower colon. It was administered in pellet form for three consecutive days in doses of 100 mg./4.5 kg. of estimated body weight. These pellets were inserted into ground meat to be consumed by the infected animals.

Fecal specimens of the animals to be treated with piperazine adipate were collected and microscopically examined for three consecutive days

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prior to treatment. Piperazine was administered in capsule form inserted in ground food in daily doses of 450 mg./4.5 kg. of estimated body weight. The treatment was continued for three consecutive days. Close observations were made to be sure that the animals had consumed the drug. After treatment, fecal specimens were examined for three days to determine the extent of parasitism.

Animals that were to be treated with dithiazanine iodide underwent a three-day fecal examination. The drug was administered in powder form sprinkled over the animals' food for 18 consecutive days. Daily dosage was 84 mg. of drug for each 4.0 to 4.5 kg. estimated body weight or 200 mg. for each 9.0 kg. estimated body weight. Fecal specimens were examined after treatment for three consecutive days to determine the extent of parasitism.

To determine the efficacy of each drug, microscopic examinations were made of feces of animals that showed positive parasitic results for three days before treatment, during the entire treatment, and three days after treatment. All side effects and reactions of animals, if any, were recorded during this survey.

TABLE 2—Occurrence and Distribution of Parasites

Animal	Infection	Total
Capuchin (*Cebus albifrons*)	Strongyloides	1
Capuchin (*Cebus capucina*)	Strongyloides	1
Chacma baboon (*Papio porcarius*)	Trichuris	2
Diana monkey (*Lasiopyga diana*)	Strongyloides	3
Gibbon (*Hylobates lar*)	Trichuris	1
Patas monkey (*Erythrocebus kerstingi*)	Trichuris	1
Orangutan (*Pongo pygmaeus*)	Strongyloides and Trichuris	2
Silver langur (*Pygathrix cristata*)	Trichuris	1
Spot-nosed monkey (*Cercopithecus nictitans*)	Trichuris	1
Yellow baboon (*Papio cyanocephalus*)	Trichuris	1
Cheetah (*Acinonyx jubatus*)	Toxocara	2
Jaguar (*Felis onca*)	Coccidia	1
Lion (*Felis leo*)	Toxocara	2
Puma (*Felis concolor*)	Toxocara	2
Snow leopard (*Felis uncia*)	Toxocara	1
Tiger (*Felis tigris*)	Toxocara	3
African porcupine (*Hystrix cristata*)	*Welcomia evoluta*	2
Algerian hedgehog (*Erinaceus algirus*)	Toxocara	3
Armadillo (*Dasypus novemcinctus*)	Unidentified ova	2
Kaffir cat (*Felis ocreata*)	Toxocara	1
Bactrian camel (*Camelus bactrianus*)	Strongyloides	1
Domestic cats (*Felis domesticus*)	Toxocara	2*
European brown bear (*Ursus arctos*)	*Ascaris transfuga*	2
Zebra (*Equus burchelli*)	*Parascaris equorum*	2
Blue racer (*Coluber constrictor*)	Unidentified ova	1
Fox snake (*Elaphe vulpina*)	Unidentified ova	1
Rhinoceros viper (*Bitis nasicornis*)	*Armillifer armillatus*	1**
Ground hornbill (*Bucorvus abyssinicus*)	Unidentified ova	1
Purple jay (*Cyanocarax affinix*)	Unidentified nematodes	1**
Cock-of-the-rock (*Rupicola rupicola*)	Unidentified ova and mites	1

* One domestic cat (*Felis domesticus*) died during this survey from food poisoning before medication was started.

** The rhinoceros viper (*Bitis nasicornis*) and the purple jay (*Cyanocarax affinix*) died and necropsies were performed.

RESULTS AND DISCUSSION

The incident rate of infection (table 1) among 391 animals observed was 13.0 per cent. This was determined from examinations of fecal specimens and necropsies of animals that died during this investigation.

The animals infected are listed (table 2).

During this survey, it was observed that the occurrence and distribution of parasites (table 2) was by a single nematode except in the orangutans (*Pongo pygmaeus*) and cock-of-the-rock (*Rupicola rupicola*). In the former host, Strongyloides and Trichuris were found; whereas, in the latter, mites and unidentified ova were observed.

Two pumas (*Felis concolor*) were the first of the animals that showed positive parasitic results to be treated with hexylresorcinol. Severe diarrhea and vomiting resulted, so medication was discontinued. Further treatments using hexylresorcinol were not attempted on other animals in this experiment.

The next anthelmintic to be used was piperazine adipate. The infection incidence before and after treatment was tabulated (table 3).

The final anthelmintic to be used was dithiazanine iodide. The infection incidence before and after treatment with dithiazanine iodide was tabulated, also. (table 4).

Jaskoski and Williamson[4] observed an incidence of 53.7 per cent nematode infection of some 400 animals examined at the Chicago Zoological Park. Jaskoski,[5] in a somewhat less extensive survey at the Lincoln Park Zoological Gardens, determined the nematode and coccidian infec-

TABLE 3—Incidence of Infection Before and After Treatment with Piperazine Adipate

Animal	Pretreatment egg count/4 Gm. feces	Post-treatment egg count/4 Gm. feces
1. Capuchin	20 Strongyloides	Negative
2. Capuchin	20 Strongyloides	Negative
3. Chacma baboon	100 Trichuris	50 Trichuris
4. Chacma baboon	100 Trichuris	50 Trichuris
5. Diana monkey	25 Strongyloides	Negative
6. Diana monkey	25 Strongyloides	Negative
7. Diana monkey	25 Strongyloides	Negative
8. Gibbon	10 Trichuris	Negative
9. Orangutan	600 Strongyloides and 50 Trichuris	100 Strongyloides and 50 Trichuris
10. Orangutan	600 Strongyloides and 50 Trichuris	100 Strongyloides and 50 Trichuris
11. Patas monkey	100 Trichuris	15 Trichuris
12. Silver langur	150 Trichuris	Negative
13. Spot-nosed monkey	10 Trichuris	25 Trichuris
14. Yellow baboon	25 Trichuris	25 Trichuris
15. Cheetah	50 Toxocara	Negative
16. Cheetah	50 Toxocara	Negative
17. Jaguar	100 Coccidia	Negative
18. Lion	100 Toxocara	Negative
19. Lion	100 Toxocara	Negative
20. Puma	25 Toxocara	Negative
21. Puma	25 Toxocara	Negative
22. Snow leopard	50 Toxocara	Negative
23. Tiger	100 Toxocara	Negative
24. Tiger	200 Toxocara	Negative
25. Tiger	250 Toxocara	Negative
26. African porcupine	400 *Welcomia evoluta*	Negative
27. African porcupine	400 *Welcomia evoluta*	Negative
28. Armadillo	50 Unidentified ova	Negative
29. Armadillo	50 Unidentified ova	Negative
30. Kaffir cat	300 Toxocara	Negative
31. Domestic cat	100 Toxocara	10 Toxocara
32. Grizzly bear	50 *Ascaris transfuga*	Negative
33. Grizzly bear	50 *Ascaris transfuga*	Negative

tion to be 20.8 per cent. The latter survey involved 477 fecal samples from 269 animals; 60 animals were infected. In the current investigation (1958–1959), 564 fecal samples were observed from 391 animals. Only 52 of the animals were infected, a reduction in infection to 13.0 per cent. It is reasonable to assume that the treatment regimen was at least largely responsible for this reduction.

The environmental differences between conditions at the Chicago Zoological Park and the Lincoln Park Zoological Gardens perhaps are factors in the significant difference in incidence of nematodiases in the two zoos. Many animals at the Chicago Zoological Park are allowed spacious freedom in outdoor areas. Anthelmintics were not administered routinely, but were given when egg count or other analyses indicated necessity of treatment.

The animals are maintained in much closer quarters at the Lincoln Park Zoological Gardens. There is usually no access to large outside areas. This closer confinement may be a factor in a decreased infection, since complete and regular removal of feces is facilitated. A further factor concerns the routine administration of phenothiazine to many of the hoofed animals at the Lincoln Park Gardens. This drug had been given for several months prior to June, 1958. At that time, treatment was interrupted when several animals (donkeys, guanacos, gnus, llamas) developed a pigmented (red) urine. In addition to the phenothiazine treatment of certain animals under a planned regimen, piperazine citrate was also used in treating others, although the regimen was not precisely planned.

The use of a coated preparation of hexylresorcinol was an attempt to prolong action of the drug by retardation of digestive enzyme action. It was administered in pellet form for three consecutive days in doses of 100 mg./4.5 kg. of estimated body weight of the animals. These pellets were inserted into ground meat to be consumed

TABLE 4—Incidence of Infection Before and After Treatment with Dithiazanine Iodide

Animal	Pretreatment egg count/4 Gm. feces	Post-treatment egg count/4 Gm. feces
1. Chacma baboon	50 Trichuris	10 Trichuris
2. Chacma baboon	50 Trichuris	10 Trichuris
3. Orangutan	100 Strongyloides and 50 Trichuris	20 Strongyloides and 10 Trichuris
4. Orangutan	100 Strongyloides and 50 Trichuris	20 Strongyloides and 10 Trichuris
5. Patas monkey	15 Trichuris	Negative
6. Spot-nosed monkey	25 Trichuris	Negative
7. Yellow baboon	25 Trichuris	Negative
8. Algerian hedgehog	10 Trichuris	Negative
9. Algerian hedgehog	10 Trichuris	Negative
10. Algerian hedgehog	10 Trichuris	Negative
11. Bactrian camel	700 Strongyloides	200 Strongyloides
12. European brown bear	25 *Ascaris transfuga*	Negative*
13. European brown bear	25 *Ascaris transfuga*	Negative*
14. Zebra	25 *Parascaris equorum*	Negative
15. Zebra	25 *Parascaris equorum*	Negative

* After 15 days of treatment, both European brown bears *(Ursus arctos)* developed severe diarrhea and vomiting. Medication was discontinued.

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by the infected animal. Two pumas *(Felis concolor)* were treated with this drug. Because severe diarrhea and vomiting occurred, medication was discontinued. Further treatments using hexylresorcinol were not attempted on other animals in this experiment, so evaluation of the anthelmintic is inconclusive.

Before administering piperazine adipate, feces of the 33 animals to be treated were examined for three consecutive days. Piperazine was administered in capsule form in daily doses of 450 mg./4.5 kg. of estimated body weight of the animal. It was given for three consecutive days in ground food to the animal, and observations were made to be sure that the drug had been consumed. After treatment, fecal specimens were examined microscopically for three consecutive days to determine the extent of parasitism; 24 were negative. The efficacy of the drug can be estimated to be 72.72 per cent. Six of the animals showed a decrease in ova count, 2 showed neither a decrease or increase in ova count, and 1 animal showed an increase in ova count. In this case, it was concluded that the parasites were either not mature enough to deposit as many ova as later on, or they may have been in the encysted form. No toxic effects or other side effects were observed in the animals treated.

For three consecutive days, fecal specimens of animals that were to be treated with dithiazanine iodide were microscopically examined. The drug was administered in powder form, sprinkled over the food, for 18 consecutive days. Daily dosage was 84.4 mg. of drug for animals weighing 4.0 to 4.5 kg., or 200 mg. of drug for each 9.0 kg. of estimated body weight. After treatment, fecal specimens were examined for three consecutive days for parasite ova. Of 15 animals treated with dithiazanine iodide, ten showed negative results. The efficacy was estimated to be about 66.6 per cent. Five of the animals showed a decrease in ova count. After 15 days of treatment, both European brown bears *(Ursus arctos)* developed severe diarrhea and vomiting; medication was discontinued.

A further survey of the treated animals is planned in the fall of 1959. This survey should reveal more information regarding the efficacy of treatment. The efficacy of dithiazanine iodide in treatment of nematodiases as revealed in this investigation is somewhat lower than that reported by most other investigators. We plan to continue the use of this anthelmintic in 1960–1961.

SUMMARY

1) An incidence of 13.0 per cent parasitic infection was found in a survey of 391 captive animals at the Lincoln Park Zoological Gardens and the Indian Boundary Zoo in Chicago.

2) Evaluation of a coated preparation of hexylresorcinol as an anthelmintic was inconclusive due to the development of diarrhea and vomiting in the puma *(Felis concolor)*.

3) Piperazine adipate is a well-tolerated, easily administered anthelmintic with an efficacy of 72 per cent in this survey.

4) Evaluation of dithiazanine iodide as an anthelmintic revealed side reactions of diarrhea and vomiting, in some cases, and an efficacy of 66 per cent.

4) Possible explanation is presented for the difference in incidence of parasites from two zoos in the same area.

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SUMMARIO IN INTERLINGUA

Le Incidentia e le Tractamento de Parasitos in Un Parco Zoologic

1. Un incidentia de 12,85 pro cento de infectiones parasitic esseva trovate in un investigation de 391 animales in captivitate al Jardines Zoologic del Parco Lincoln e al Parco Zoologic Indian Boundary a Chicago.

2. Le evalutation de un drageificate preparato de hexylresorcinol como antihelmintico esseva inconclusive a causa del disveloppamento de diarrhea e vomito in le puma *(Felis concolor)*.

3. Adipato de piperazina es un ben-tolerate e facilemente administrabile antihelmintico, con un efficacia de 72 pro cento secundo le datos del presente investigation.

4. Le evalutation de ioduro de dithiazanina como antihelmintico revelava le adverse effectos secundari de diarrhea e vomito in certe casos. Su efficacia esseva 66 pro cento.

5. Es presentate un explication possibile pro le differentia inter le incidentias de parasitos in duo parcos zoologic in le mesme area.

Correction—A Comparison of the Effects of Four Strains of Newcastle Disease Virus

In the article, "A Comparison of the Effects of Four Strains of Newcastle Disease Virus on a Strain of Human Epithelial-like Cells (Maben) and Strain L Mouse Fibroblasts," by P. Subramanyam and B. S. Pomeroy, in the January, 1960, issue of the **American Journal of Veterinary Research** (p. 134), the legend for figure 1 should read:

"Cytopathogenic changes observed 24 hours after inoculation of various strains of Newcastle disease virus into Maben cell cultures, shown on the glass walls of test tubes. (A) GB Texas strain—most of the cells have fallen off the glass and the cells remaining on the glass are swollen, shrunken, or appear as cell debris; (B) Kan. Manhatten strain—cells show swelling and fragmentation and many cells have fallen off the glass; (C) NJ-Roakin—the effect is the same as the Manhatten strain but to a milder degree; (D) Bl—cells appear shrunken and few have fallen off the glass; (E) normal cells. x 250.

The Protein Nature of the Matrices of Negri Bodies

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In 1932, Covell and Danks [7] reported a series of histochemical studies on the nature of Negri bodies. They were able to demonstrate the presence of deoxyribonucleic acid (DNA) and probably tyrosine at the sites of the basophilic granules, within the inclusions, by the Feulgen and Millon techniques, respectively. They also showed that the inclusion bodies contain masked iron and leave an ash residue upon micro-incineration.

Caspersson [6] (1950), in discussing the simple systems for protein formation within cells, categorized rabies virus, by means of the Feulgen technique, among those viruses containing DNA during reproduction. In 1952, Wolman and Behar [19] reported that as the Negri body ages and changes from a basophilic to an amorphous acidophilic structure, it loses its Feulgen positivity. In addition, they showed that the Feulgen-positive inclusions gave a positive reaction for alkaline phosphatase and cholinesterase and were weakly positive for lipase. The intensity of these latter reactions diminished in proportion to the decrease in basophilic and Feulgen positivity.

Also in 1952, Johnson [13] indicated that the absence of DNA in Negri bodies conformed with the known characteristics of inclusion bodies associated with some other viruses. He also stated that the matrix of the Negri body contains considerable quantities of a lipid material. Moulton,[15] in 1954, reported an extensive histochemical study of Negri bodies. Using conventional staining procedures, he was able to demonstrate the presence of protein, *alpha*-amino acid, arginine, tyrosine and, inconstantly, DNA within the inclusions. He presumed that the inclusions, which were negative for DNA, were older Negri bodies. He obtained inconclusive results with the periodic acid-Schiff reaction for the polysaccharide complexes. Using applicable staining techniques, he reported negative results for attempts to demonstrate ribonucleic acid, glycogen, mucopolysaccharides, ascorbic acid, lipids, phospholipids, cholesterol, inorganic iron, organic iron, calcium, alkaline phosphatase, dehydrogenase, and cytochrome oxidase.

From a review of the literature, it is apparent that Negri bodies contain substances of a protein nature as demonstrated by gross biochemical techniques. In recent years, studies in the field of protein histochemistry have led to the development of several methods for the identification of certain reactive protein groups in tissue sections.[2-5, 8, 9, 11, 18] The study reported here was undertaken in an attempt to more specifically characterize the protein nature of Negri bodies by such histochemical methods.

Materials and Methods

Paraffin-embedded blocks of formalin-fixed brain tissues from 4 cows and 2 cats which died of rabies were furnished by Dr. Frank Ramsey, Department of Veterinary Pathology, Veterinary Division, Iowa State College, Ames. All of these animals died in 1958 and a diagnosis of rabies was established by animal inoculation or the observation of Negri bodies in suitably stained microscopic tissue sections, or both. In addition, paraffin-embedded blocks of Zenker-fixed brain tissue from 5 dogs that died of rabies were furnished by Dr. Wayne Anderson of the Agricultural Research Service, USDA, Denver, Colo. All of these dogs died approximately ten years previously and the diagnosis of rabies was based on the observation of Negri bodies in suitably stained microscopic tissue sections.

Sections, 6 to 8 μ in thickness, were prepared from each block of paraffin-embedded tissue furnished for this study. Each section was stained by the Schleifstein stain for Negri bodies.[12] These sections were then examined by conventional bright-field microscopy. One block of tissue from each animal was selected on the basis of the abundance of Negri bodies for use in further histochemical studies. Serial sections, 6 to 8 μ in thickness, were cut from the selected blocks and mounted on glass microscope slides without the use of an adhesive.

The serial sections from each animal were stained by the following techniques and examined by conventional bright-field microscopy. Prior to the staining of the Zenker's acetic-fixed tissue, the sections were treated by rinsing for two minutes in an 0.25 per cent alcoholic iodine solution (w/v) followed by a second rinse for an equal period of time in an aqueous 5 per cent sodium thiosulfate solution (w/v).

From the Pathology Branch, Research Support Division, United States Army Medical Research and Nutrition Laboratory, Fitzsimons Army Hospital, Denver, Colo.

The photographs were taken by Mr. William Hummel of the Pathology Service, Fitzsimons Army Hospital, Denver, Colo. The authors appreciate the technical assistance of Private Herbert S. Yamanaka and Miss Nancy Fore of the Laboratory Section of the Pathology Branch, Research Support Division, United States Army Medical Research and Nutrition Laboratory.

Demonstration of Cell Structure and Negri Bodies.—Single sections for each animal were stained with Schleifstein stain and Harris' hematoxylin and aqueous eosin.[12] With respect to the latter stain, the sections were intentionally overstained with eosin.

Demonstration of Sulfhydryl Groups, Disulfide Linkages, Tyrosine, and Amino Groups.—Single sections from each animal were stained with tetrazotized benzidine coupled with *beta*-naphthol, according to the procedure given by Lillie for diazo reactions,[14] and the same method was modified by staining at 25 C., which reduced the time prescribed for each step by 50 per cent, and by counterstaining with Harris' hematoxylin. In addition, paired tissue sections from each animal were stained with dinitrofluorobenzene (DNFB) coupled with H acid or *beta*-naphthol.[8, 15] In this and subsequent procedures in which H acid or *beta*-naphthol was coupled with DNFB, Harris' hematoxylin was used as a counterstain.

Specific Demonstration of Sulfhydryl Groups (Cysteine).—Paired sections from each animal were treated with diazoaniline,[13] followed by treatment with nitrous acid.[16] One section was stained with DNFB coupled with H acid and the other with DNFB coupled with *beta*-naphthol. In addition, one section from each animal was washed with running tap water for 90 minutes to liberate the sulfhydryl groups [16] and stained with 2,2′-dihydroxy-6,6′-dinaphthyl disulfide (DDD).[2, 4, 5]

Specific Demonstration of Disulfide Linkages (Cystine).—Three sections from each animal were treated with benzoyl chloride and the cystine reduced with hydrocyanic acid.[13] One section was stained with DNFB coupled with H acid, the second with DNFB coupled with *beta*-naphthol and the third with the DDD stain.

Specific Demonstration of Amino Groups (Lysine or Terminal Amino).—Paired sections from each animal were treated with hydrogen peroxide [13] followed by treatment with diazoaniline. One section was stained with DNFB coupled with H acid and the other with DNFB coupled with *beta*-naphthol.

Specific Demonstration of Tyrosine.—Paired sections from each animal were treated with hydrogen peroxide followed by treatment with nitrous acid. One section was stained with DNFB coupled with H acid and the other with DNFB coupled with *beta*-naphthol.

Demonstration of Nucleic Acids.—Single sections from each animal were stained by the gallocyanin-chrome alum method for nucleic acids,[16] and the nuclear Feulgen reaction for DNA.[14]

Demonstration of Reduction Sites.—Upon the advice of Dr. Frank Johnson, Chief, Histochemistry Branch of the Armed Forces Institute of Pathology, selected tissue sections from each animal were subjected to the ferric ferricyanide reduction test.[14]

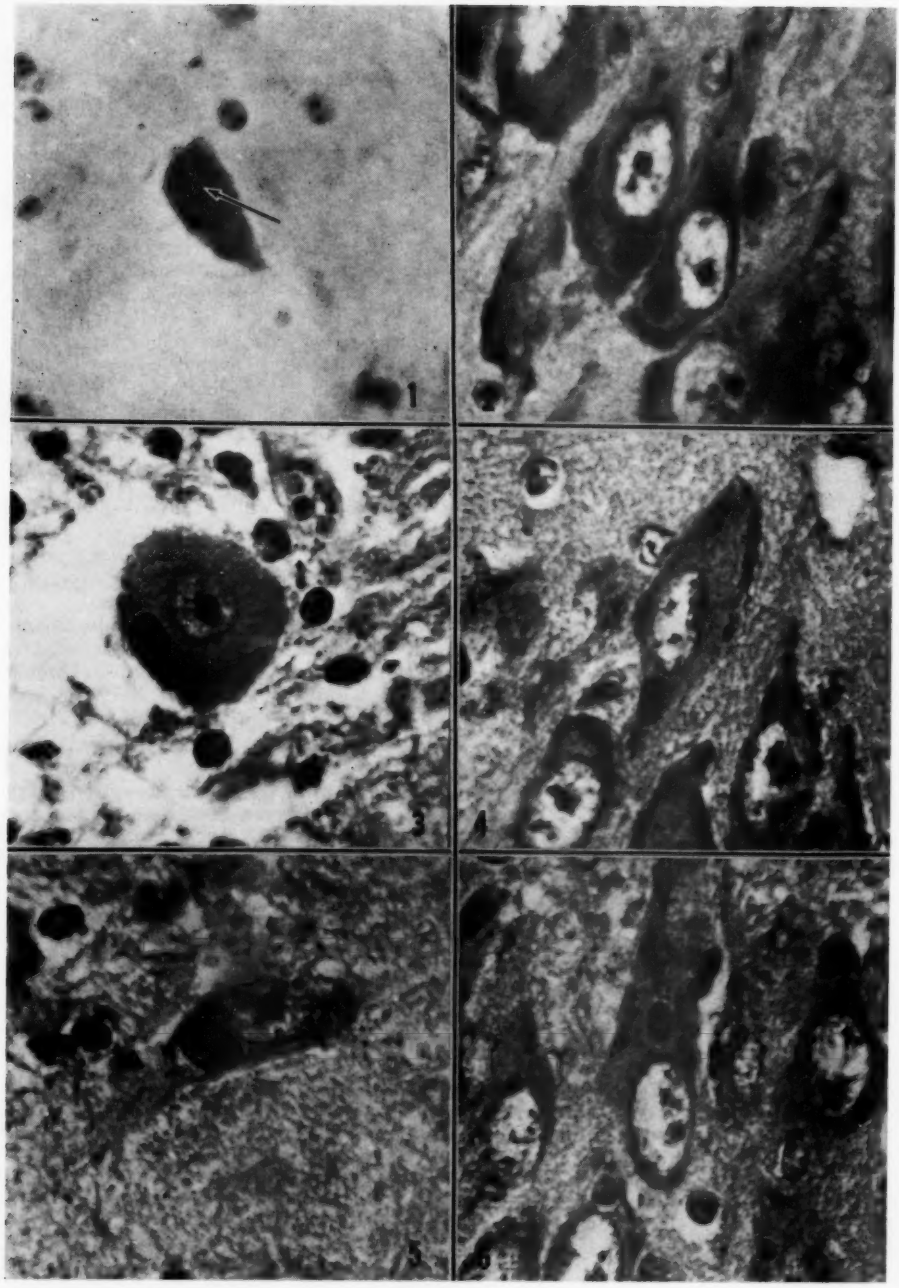
RESULTS

In the tissue sections stained with Schleifstein's stain, the size of the Negri bodies varied from case to case and between neurons of a single case. In the cattle, where the cerebellum was more often available for sectioning, they occurred most frequently within the cytoplasm of Purkinje cells. In the dogs and cats, where the cerebrum was the principal tissue examined, the Negri bodies occurred most frequently within the cytoplasm of the neurons in the hippocampus. Although the Negri bodies stained more intensely in the Zenker-fixed tissue, they stained strongly enough in the formalin-fixed tissue to be detected. They exhibited a magenta-colored matrix which contained faintly basophilic inner granules recognizable at high-dry magnification (fig. 1, 2). In the sections stained with Harris' hematoxylin and overstained with aqueous eosin, the Negri bodies, regardless of their size, had faintly staining, amorphous, acidophilic matrices which contained basophilic granules. Except with reduced illumination, the Negri bodies did not contrast strongly with the acidophilic cytoplasm of the cells in which they occurred.

The results obtained from the histochemical procedures employed to demonstrate the reactive groups of protein and nucleic acids were uniform. Therefore, the staining reaction of the Negri bodies can be reported without reference to the fixative used, the case in which they occurred, or the location of the involved neurons (table 1). The characteristics of each stain were as follows:

Tetrazotized Benzidine Coupled with Beta-Naphthol.—With both the procedure given by Lillie and the modification previously described, the matrices of the Negri bodies were red to orange. They exhibited basophilic granules within their matrices. The cytoplasm of the neurons was orange to orange-brown, but was fainter in color than the Negri bodies (fig. 3, 4). The modified procedure gave slightly less vivid colors than Lillie's method.

Dinitrofluorobenzene (DNFB) *Coupled with Either H Acid or Beta-Naphthol.*—Negri bodies had light tan to brown matrices which contained basophilic inner granules. The color reaction of the inclusions was relatively constant within the same tissue section but varied in intensity between sections. The neuronal cytoplasm stained light brown. The *beta*-naphthol gave a more intense color reaction than did the H acid. This latter observation was true



Figures 1-6

in all instances where the two agents were used.

Sulfhydryl Groups (DNFB).—In sections treated by diazoaniline to block tyrosyl groups and by nitrous acid to block amino groups and stained with DNFB coupled with either H acid or *beta*-naphthol, the Negri bodies had tan to brown matrices which contained basophilic granules (fig. 5, 6). The neuronal cytoplasm gave a similar, though less intense, reaction.

Sulfhydryl Groups (DDD).—In tissue sections stained with the DDD stain, the Negri bodies had violet matrices. The inner granules observed in the matrices of the Negri bodies when stained with the DDD methods had a variable reaction in that some appeared as vacuoles and others as dark blue bodies. The neuronal cytoplasm gave a similar, though less intense, reaction.

Disulfide Linkages (DNFB).—In sections treated by benzoyl chloride to block tyrosyl, amino, and sulhydryl groups, treated with hydrocyanic acid to reduce the cystine, and stained with DNFB coupled with either H acid or *beta*-naphthol, the Negri bodies exhibited light brown to violet matrices which contained basophilic granules (fig. 7, 8). The neuronal cytoplasm gave a similar, though usually less intense, reaction; however, in some cases it was so intense that the Negri bodies did not contrast readily.

Disulfide Linkages (DDD).—In sections treated by benzoyl chloride to block the tyrosyl, amino, and sulfhydryl groups, and with hydrocyanic acid to reduce the cystine, and stained with the DDD stain for the demonstration of disulfide linkages, the color of the Negri bodies and neuronal cytoplasm was identical to that seen in the sections stained for sulfhydryl groups (DDD).

Amino Groups (DNFB).—In sections treated with hydrogen peroxide to block sulfhydryl groups, diazoaniline to block tyrosyl groups, and stained with DNFB coupled with either H acid or *beta*-naphthol, the Negri bodies had tan to dark brown matrices which contained basophilic granules (fig. 9, 10). The cytoplasm of the neurons was faint yellow to tan.

Tyrosyl Groups (DNFB).—In sections treated with hydrogen peroxide to block sulfhydryl groups and nitrous acid to block amino groups, and stained with DNFB coupled with either H acid or *beta*-naphthol, the Negri bodies had tan to brown matrices which contained basophilic granules (fig. 11, 12). The neuronal cytoplasm was faint yellow to tan.

Nucleic Acids.—In sections stained by the nuclear Feulgen reaction, the matrices and inner granules of the Negri bodies and the neuronal cytoplasm remained unstained in the tissue from all 11 animals. In sections from the tissue of all 11 animals stained by the gallocyanin-chrome alum method, the matrices appeared to be light blue in contrast to the dense basophilia of the cytoplasm. Occasionally the inner granules appeared as indistinct, weakly basophilic bodies.

Ferric Ferricyanide Reduction Sites.—In sections subjected to the ferric ferricyanide reduction test, the matrices of the Negri bodies acted as reduction sites and stained deep blue. The inner basophilic granules were discernible and did not act as reduction sites.

Legends for Figures 1-6 on Opposite Page

Fig. 1—Negri body in a neuron of the hippocampus of an ox. Formalin fixation. Schleifstein's stain; x 1,425.

Fig. 2—Negri bodies in neurons of the hippocampus of a dog. Zenker's acetic fixation. Schleifstein's stain; x 1,425.

Fig. 3—Negri body in a Purkinje cell of the cerebellum of an ox. Formalin fixation. Section stained with tetrazotized benzidine coupled with beta-naphthol to demonstrate tyrosine, histidine, and tryptophan; x 1,425.

Fig. 4—Negri body in a neuron of the hippocampus of a dog. Zenker's acetic fixation. Section stained with tetrazotized benzidine coupled with beta-naphthol to demonstrate tyrosine, histidine, and tryptophan. Hematoxylin counterstain; x 1,425.

Fig. 5—Negri body in a neuron of the hippocampus of an ox. Formalin fixation. Section treated by diazoaniline to block tyrosyl groups, and nitrous acid to block amino groups. The dinitrofluorobenzene stain coupled with beta-naphthol was employed to demonstrate the presence of sulfhydryl groups. Hematoxylin counterstain; x 1,425.

Fig. 6—Negri bodies in the neurons of the hippocampus of a dog. Zenker's acetic fixation. Section treated by diazoaniline to block tyrosyl groups, and nitrous acid to block amino groups. The dinitrofluorobenzene stain coupled with H acid was employed to demonstrate the presence of sulfhydryl groups. Hematoxylin counterstain; x 1,425.

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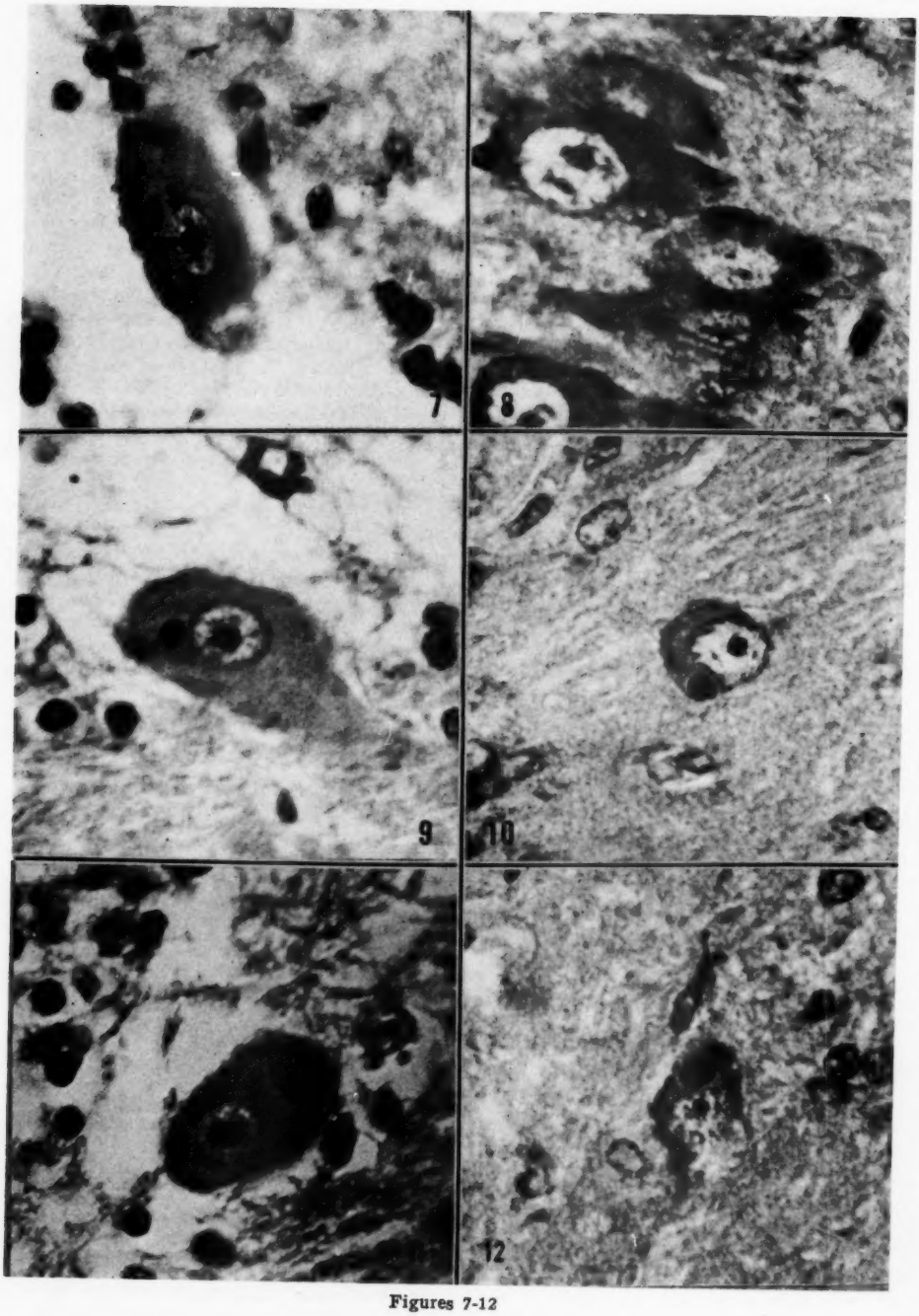

Figures 7-12

DISCUSSION

The results obtained in this study confirm the previously reported [6,7,15,19] protein nature of Negri bodies. The specific reactive groups of protein within these inclusions have not been previously reported, with the exception of *alpha*-amino [15] and tyrosyl groups.[7,15] Variable quantities of these specific protein groups are thought to be in an available reactive form prior to staining,[3] and not appreciably altered by the formalin or Zenker's fixatives [1,16] used in this study. It is quite probable that a weakly acid mercurial fixative such as Zenker's acetic solution, in which the mercury would react with the sulfhydryl groups of some of the cysteine, could reduce the quantity of this reactive group available for staining. At the same time it would increase the number of disulfide linkages available for staining.[1] However, any such change in various reactive groups incident to fixation, unless of major magnitude, would not be readily detected by the methods used in this study, since they indicate the presence of, but not the relative abundance of, these groups.

The results obtained with Harris' hematoxylin overstained with aqueous eosin indicate that the Negri bodies observed in the tissues used in this study were acidophilic. According to Wolman and Behar,[19] the acidophilic nature of the inclusions indicates that they are relatively old Negri bodies. Young forms of such inclusions are basophilic.[19] The actual age of the Negri bodies observed in each case cannot be ascertained, since all the tissues were from animals that had died of rabies. The results obtained with the hematoxylin and eosin stain indicate that we were dealing with old inclusion bodies, which would be expected in animals dead of rabies.

The positive reaction of the matrices of these inclusions, when stained with tetrazotized benzidine coupled with *beta*-naphthol, indicates the presence of any or all of the following substances: histidine, tryptophan, and tyrosyl groups.[14] The presence of sulfhydryl groups, disulfide linkages, tyrosine, and amino groups are indicated by the positive reaction to the DNFB stain.[9] These reactive groups of proteins were individually demonstrated by appropriate blocking agents and staining by DNFB coupled with either H acid or *beta*-naphthol. In addition, the presence of sulfhydryl groups and disulfide linkages within the matrices of these inclusions was substantiated by the positive reaction of the inclusions to the DDD stains.[3]

We were unable to demonstrate the presence of nucleic acids in general or DNA specifically in any Negri body. According to Wolman and Behar,[19] Negri bodies observed in young mice within the first six days after experimental infection gave a strong Feulgen-positive reaction. This reaction decreased in intensity as the inclusions aged and disappeared as they became

Legends for Figures 7-12 on Opposite Page

Fig. 7—Negri bodies in a Purkinje cell of the cerebellum of an ox. Formalin fixation. Section treated by benzoyl chloride to block tyrosyl, amino, and sulfhydryl groups, and treated with hydrocyanic acid to reduce cystine. The dinitrofluorobenzene stain coupled with beta-naphthol was employed to demonstrate the presence of disulfide linkages. Hematoxylin counterstain; x 1,425.

Fig. 8—Negri bodies in neurons of the hippocampus of a dog. Zenker's acetic fixation. Section treated by benzoyl chloride to block tyrosyl, amino, and sulfhydryl groups, and treated with hydrocyanic acid to reduce cystine. The dinitrofluorobenzene stain coupled with H acid was employed to demonstrate the presence of disulfide linkages. Hematoxylin counterstain; x 1,425.

Fig. 9—Negri body in a Purkinje cell of the cerebellum of an ox. Formalin fixation. Section treated by hydrogen peroxide to block sulfhydryl groups, and diazoaniline to block tyrosyl groups. The dinitrofluorobenzene coupled with H acid was employed to demonstrate the presence of amino groups. Hematoxylin counterstain; x 1,425.

Fig. 10—Negri body in a neuron of the hippocampus of a dog. Zenker's acetic fixation. Section treated by hydrogen peroxide to block sulfhydryl groups, and diazoaniline to block tyrosyl groups. The dinitrofluorobenzene stain coupled with H acid was employed to demonstrate the presence of amino groups. Hematoxylin counterstain; x 1,425.

Fig. 11—Negri body in a Purkinje cell of the cerebellum of an ox. Formalin fixation. Section treated by hydrogen peroxide to block sulfhydryl groups, and nitrous acid to block amino groups. The dinitrofluorobenzene stain, coupled with beta-naphthol was employed to demonstrate tyrosyl groups. Hematoxylin counterstain; x 1,425.

Fig. 12—Negri body in a neuron of the hippocampus of a dog. Zenker's acetic fixation. Section treated by hydrogen peroxide to block sulfhydryl groups, and nitrous acid to block amino groups. The dinitrofluorobenzene stain, coupled with H acid was employed to demonstrate tyrosyl groups. Hematoxylin counterstain; x 1,425.

TABLE 1—Staining Characteristics of Negri Bodies

Stain	Staining reaction of Negri body
Tetrazotized benzidine coupled with *beta*-naphthol	Matrix, positive
Dinitrofluorobenzene (DNFB) coupled with either H acid or *beta*-naphthol	Matrix, weak to moderate positive
Sulfhydryl (DNFB) coupled with either H acid or *beta*-naphthol	Matrix, positive
Sulfhydryl (DDD)	Matrix, positive
Disulfide (DNFB) coupled with either H acid or *beta*-naphthol	Matrix, weak to moderate positive
Disulfide (DDD)	Matrix, positive
Amino (DNFB) coupled with either H acid or *beta*-naphthol	Matrix, weak to moderate positive
Tyrosyl (DNFB) coupled with either H acid or *beta*-naphthol	Matrix, positive
Gallocyanin-chrome alum for nucleic acids	Matrix and inner granules negative
Nuclear Feulgen for DNA	Matrix and inner granules negative
Ferric ferricyanide reduction test	Matrix, positive

acidophilic. Considering that all of the Negri bodies we observed were acidophilic, it could be expected that no positive reaction for DNA would be encountered. In addition, while neither the matrices nor the inner granules gave a positive Feulgen reaction, the granules themselves often appeared as dark blue bodies in the DDD stain where no hematoxylin counterstain was employed. In all the other stains where hematoxylin was employed they were basophilic. These results do not permit an interpretation of the presence or absence of reactive groups of protein in the inner granules.

The consistent demonstration of sulfhydryl groups (cysteine), disulfide linkages (cystine), tyrosine, and amino groups (lysine or terminal amino) in the matrices of Negri bodies indicates that proteins or polypeptides are present. These data confirm, in part, the report of Covell and Danks [7] that Negri bodies contain tyrosine. They agree, in part, with the work of Moulton,[15] who demonstrated tyrosine, *alpha*-amino acids, and unspecified protein in Negri bodies. Our observation that they contain sulfhydryl and disulfide groups, which has not been reported previously, further demonstrates the protein nature of Negri bodies, since these two reactive groups are characteristic of polypeptides and proteins in general.

The presence of sulfhydryl and tyrosyl groups in the matrices of Negri bodies affords a known chemical substance for which a color reaction can be developed if the tissue has been fixed in formalin, Zenker's acetic solution, or absolute alcohol. Difficulty is frequently encountered when the histologic demonstration of Negri bodies by conventional stains, such as Schleifstein's stain, is attempted on tissues fixed in other than mercurial sublimate fixatives. In such instances, the use of a suitable stain for sulfhydryl or tyrosyl groups in tissue sections would be of value in the detection of Negri bodies. Of the various protein stains which we have used, the tetrazotized benzidine-*beta*-naphthol stain performed according to the method of Lillie,[14] or as modified by this laboratory, or the DNFB-*beta*-naphthol stain would be most suitable for this purpose. These stains are of broad spectrum and impart a more vivid color to proteins than any of the stains used for demonstration of specific reactive protein groups. Hematoxylin counterstain may prove valuable, as it stains the nuclei of neurons and glial cells and thereby aids in distinguishing cell types.

The ferric ferricyanide reduction test should also be useful in the staining of Negri bodies. The tyrosine and cysteine present in these inclusions are both capable of reducing ferricyanide to ferrocyanide which is converted to insoluble Prussian blue.[14]

In our study, the nucleoplasm of the neurons did not contain any of the reactive protein groups observed in the Negri bodies. The chromatin of the neurons frequently contained beads or foci which gave positive reactions for the various protein groups as demonstrated in the Negri body, in addition to nucleic acid as demonstrated by the hematoxylin counterstain, the gallocyanin-chrome alum stain, and the nuclear Feulgen reaction. The affinity of the nucleoli for the hematoxylin and protein stains imparted a muddy appearance to their protein reaction. Because of this mixed staining of the nucleoli, they appeared darker than the Negri bodies. The nucleoli in neu-

rons which contained Negri bodies did not appear different from those in cells which did not contain such inclusions.

In every instance the cytoplasm of the neurons, in which Negri bodies occurred, gave the same reaction for the protein stains as the matrix of the inclusion. From these observations, it appears that the matrices of Negri bodies are similar in protein composition to the cytoplasm of neurons. Although the exact mechanism for the formation of Negri bodies is unknown, Covell and Danks [7] have expressed the thought that they probably arise as a condensation of cytoplasmic substances, or debris incident to cytolysis, around virus particles. Our observations lend additional evidence to this theory.

The conclusion drawn from this study is that the matrices of the Negri bodies contain reactive groups normally associated with the presence of proteins or polypeptides. Our results also indicate that the reactive groups are not significantly altered by the nature of the fixative used, being demonstrable in both formalin and Zenker's acetic-fixed tissues.

SUMMARY

Negri bodies observed within neurons of 4 cattle, 2 cats, and 5 dogs which died of rabies were shown to contain reactive groups normally associated with proteins or polypeptides.

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SUMMARIO IN INTERLINGUA

Le Natura Proteinic del Matrices de Corpores de Negri

Esseva demonstrate que le corpores de Negri observate in le neurones de 4 bestiales, 2 cattos, e 5 canes morte de rabie contineva gruppos reactive que es normalmente associate con proteinas o polypeptidos.

The Effect of Ovarian Hormones on the Uterine Defense Mechanism During the Early Stages of Induced Infection

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PREVIOUS STUDIES have indicated that progesterone inhibits the uterine defense mechanism.[1,2,6] Greater numbers of bacteria were recovered from experimentally infected uteri of rabbits and cows in the luteal phase than from uteri during the follicular phase or from ovariectomized rabbits and cows.[2,6]

In the previous studies, the relative numbers of bacteria alive in the uterus were determined at intervals of 24 hours or more after inoculation. It would seem possible that, in this interval, many factors besides the uterine defenses might have influenced the number of live bacteria in the uterus. If any such factors were influenced by the endocrine state of the animal, the effects of a particular hormone on the uterine defenses might have been obscured.

This study was conducted to investigate the effects of ovarian hormones on the uterine defenses during the first few hours of induced *Escherichia coli* infection. The difference in bactericidal activity of estrous and pseudopregnant rabbit uteri is evident by four hours and is actually greater at this time than at later intervals.[3,5]

EXPERIMENT 1—THE EFFECT OF OVARIAN HORMONES ON LEUKOCYTIC RESPONSE AND BACTERICIDAL ACTIVITY BY FOUR HOURS POSTINOCULATION

Animals and Methods.—Estrous, pseudopregnant, and ovariectomized rabbits were compared in the first seven replicates. In each set of replicates there were 3 rabbits, 1 in each of the three endocrine states. An estrogen-treated, ovariectomized rabbit and a progesterone-treated, ovariectomized rabbit were also included in each of five additional sets of replicates.

Estrous rabbits were in heat the day of inoculation, but were not allowed to mate. Pseudopregnant rabbits were used eight, nine, or ten days after ovulation was induced by mating to a vasectomized buck or by intravenous injection of chorionic gonadotrophin. Ovariectomized rabbits were inoculated 15 days after the ovaries were removed through bilateral flank incisions.

The ovariectomized, estrogen-treated rabbits in replicates 8 and 9 were ovariectomized while in heat and given 10 μg. of *beta*-estradiol per day for eight days; those in replicates 10, 11, and 12 were untreated for 15 days after ovariectomy, then given 10 μg. of estradiol per day for eight days. The progesterone-treated rabbits were also ovariectomized while in heat. Those in replicates 8 and 9 were given 1 mg. of progesterone per day for eight days after ovariectomy. Those in the last three replicates were untreated for 15 days following ovariectomy, then were given 10 μg. of *beta*-estradiol per day for two days, followed by 1 μg. of estradiol and 1 mg. of progesterone per day for three days and 1 mg. of progesterone daily for five additional days.

All hormones were given subcutaneously in olive oil. Uteri of the hormone-treated rabbits were inoculated the day following the last hormone injection.

The rabbits were anesthetized with sodium pentobarbital and laparotomized; one uterine horn of each rabbit was ligated at each end to prevent drainage of the bacterial inoculum from the uterine lumen. The anterior end of the uterine horn was ligated at the tubo-uterine junction, care being taken not to ligate veins coming from the uterus. The cervix was ligated through an incision in the anterior end of the vagina. Each ligated horn was inoculated by puncturing the uterine wall carefully with a 27-gauge hypodermic needle and administering 0.2 ml. of a saline solution (0.87% NaCl) suspension of *Escherichia coli* washed from trypticase soy agar slants.

The mean log number of *Esch. coli* per inoculum (table 1) was 8.28 (191,000,000 cells). The rabbits were killed four hours later and each inoculated horn was flushed aseptically with 10.0 ml. of sterile physio-

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logic saline solution. The flushings were diluted serially, plated in violet red bile agar, and incubated at 37 C. for 18 to 24 hours, when the total number of *Esch. coli* was calculated from the colony counts. The numbers of leukocytes in the flushings were estimated from microscopic sweeps of dried and stained flushings (several 0.01 ml. samples, each spread over a circular area 1 cm. in diameter).

A specimen of each uterine horn was fixed in Bouin's solution for histologic study.

Results and Discussion.—Mean log numbers of *Esch. coli* recovered from uteri of ovariectomized, estrous, and pseudopregnant rabbits in all 12 replicates were as follows (table 1): ovariectomized, 5.58 (380,000); estrous, 5.54 (347,000); and pseudopregnant, 7.92 (83,200,000). The numbers recovered from uteri of estrous and ovariectomized rabbits were similar, and each was significantly less than the number recovered from pseudopregnant rabbits ($P<0.01$ for each comparison).

The numbers of *Esch. coli* recovered from the estrogen or progesterone-treated ovariectomized rabbits were quite similar to the numbers flushed from the estrous and pseudopregnant rabbits, respectively, in the corresponding replicates. For replicates 8 through 12, the mean log numbers were as follows: ovariectomized controls, 5.44 (275,000); estrous, 6.26 (1,820,000); ovariectomized estrogen-treated, 5.33 (214,000); pseudopregnant, 7.51 (32,400,000); ovariectomized progesterone-treated, 7.24 (17,400,000).

The uterine bactericidal activity of rabbits in the various endocrine groups was related grossly to the degree of leukocytic response, an association seen previously during the first four hours of induced *Esch. coli* uterine infection.[3] The mean log number of leukocytes flushed from uteri of the 12 ovariectomized rabbits was 7.40 (25,100,000), greater ($P = 0.02$) than the number in the 12 pseudopregnant rabbits, 6.84 (6,920,000), but less ($P<0.05$) than the number in the 12 estrous rabbits, 7.94 (87,100,000). In the last five replicates, the log numbers of leukocytes for the various types of rabbits were: ovariectomized, 7.48 (30,200,000); estrous, 7.67 (46,800,000); ovariectomized estrogen-treated, 8.33 (214,000,000); pseudopregnant, 7.02 (10,500,000); and ovariectomized progesterone-treated, 6.72 (5,250,000).

The relative numbers of live *Esch. coli* flushed from uteri of rabbits in the various endocrine states indicate that progesterone inhibits the uterine bactericidal activity during the first four hours of induced *Esch. coli* infection. These results are thus in agreement with those obtained previously at later stages of induced infection.[1,6]

The pseudopregnant and ovariectomized progesterone-treated rabbits had fewer leukocytes in the uterine lumina than did rabbits in the other endocrine groups. It seems likely that progesterone inhibited uterine bactericidal activity by inhibiting the inflammatory response.

The relative numbers of leukocytes flushed from uteri of estrogen-influenced and ovariectomized rabbits suggested that

TABLE 1—Log Numbers of Escherichia coli and Leukocytes Flushed from Uteri of Rabbits in Various Endocrine States Four Hours after Experimental Inoculation

		Endocrine state of rabbit									
		Ovariectomized		Estrous		Ovariectomized; estrogen-treated		Pseudopregnant		Ovariectomized; progesterone-treated	
Replicate	Uterine inoculum	*Esch. coli.*	Leukocytes	*Esch. coli*	Leukocytes	*Esch. coli*	Leukocytes	*Esch. coli*	Leukocytes	*Esch. coli*	Leukocytes
1	8.26	7.60	6.52	4.51	8.32	.....	.....	8.57	6.79	.....	.....
2	8.25	4.87	7.88	4.71	7.95	.....	.....	8.14	6.58	.....	.....
3	8.25	5.52	6.84	5.42	7.96	.....	.....	8.19	6.48	.....	.....
4	8.16	5.93	7.45	5.56	7.61	.....	.....	8.11	6.96	.....	.....
5	8.40	5.74	8.14	5.66	8.33	.....	.....	7.05	7.51	.....	.....
6	8.24	5.33	6.54	5.79	8.25	.....	.....	8.67	6.67	.....	.....
7	8.35	4.73	8.02	3.48	8.51	.....	.....	8.76	5.98	.....	.....
8	8.27	6.24	7.05	7.61	6.70	5.49	8.32	7.32	7.14	7.02	6.32
9	8.39	5.72	7.79	6.62	7.29	5.28	8.66	6.73	7.39	8.14	6.31
10	8.28	4.40	7.40	5.78	8.01	5.67	7.83	8.60	6.51	8.08	6.52
11	8.29	6.23	7.11	6.08	7.96	4.58	8.69	7.19	7.02	6.16	7.43
12	8.23	4.61	8.07	5.21	8.41	5.64	8.14	7.72	7.06	6.81	7.04
Mean	8.28	5.58	7.40	5.54	7.94	5.33	8.32	7.92	6.84	7.24	6.72

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estrogen, either endogenous or exogenous, stimulated the leukocytic response. The response was particularly great in the ovariectomized estrogen-treated rabbits.

The estrogenic stimulation of the leukocytic response, although showing little effect on the number of *Esch. coli* killed, was associated with faster clearance of bacterial cells from the uterine lumen. Few *Esch. coli* cells were found microscopically in the uterine flushings of estrous or estrogen-injected rabbits. However, flushings from some ovariectomized rabbits—those in which leukocytic response was limited—showed the presence of large numbers of *Esch. coli* cells. Most of these cells had been killed *in utero*, since the plate counts were comparatively small.

Uterine flushings from several progesterone-influenced rabbits also had *Esch. coli* plate counts which were only a small fraction of the microscopic counts. For example, this occurred in 3 of the 5 ovariectomized progesterone-treated rabbits and 4 of the 5 pseudopregnant rabbits in the same replicates. The factors responsible for causing large discrepancies between plate and microscopic counts were probably the noncellular bactericidal substances found previously in uterine exudates.[3] Leukocytes appear to be the sole or major source of these substances,[4] which were found to have some defensive value against *Esch. coli* by killing large numbers of bacteria *in utero*. This was seen most often in pseudopregnant rabbits. As the leukocytic response continued to develop, the killed *Esch. coli* were cleared from the uterine lumina.

Larger *Esch. coli* inocula might have shown an estrogenic enhancement of uterine bactericidal activity as well as clearance of *Esch. coli* from the uterine lumen. Even so, the results of this experiment show both an inhibition of the uterine defenses by progesterone and a stimulation by estrogen. The effect of each hormone was associated with an effect on leukocytic response.

Uteri of ovariectomized rabbits varied somewhat in size at the time of inoculation, but all were considerably smaller and uterine walls much thinner than those of estrous or pseudopregnant rabbits. Uteri of estrous and ovariectomized estrogen-treated rabbits were similar in gross and microscopic appearance. Pseudopregnant and ovariectomized progesterone-injected rabbits also had similar uteri, extreme endometrial proliferation having occurred in rabbits of both groups.

EXPERIMENT 2—HORMONAL EFFECTS ON THE EARLY LEUKOCYTIC RESPONSE

The first experiment gave an indication of the hormonal influence on the number of leukocytes in the uterine lumen at the standard time interval of four hours after uterine inoculation. In this part of the study, a more thorough comparison was made of the leukocytic responses in uteri of estrous, ovariectomized, and pseudopregnant rabbits.

Experimental Material.—The rabbits represented here were those used in the previous experiment and other rabbits killed at intervals other than four hours postinoculation but used during the same period of time as those in the first experiment. The uterine sections examined were cut from specimens fixed in Bouin's solution, sectioned at 8 μ, and stained with hematoxylin and eosin.

Specimens from uninoculated uteri for comparison purposes were removed from unoperated rabbits. Specimens from inoculated uterine horns of rabbits in all three endocrine states were taken two or four hours after experimental inoculation. Specimens were taken from estrous and pseudopregnant rabbits at 16 hours and from pseudopregnant rabbits at several intervals between four and 16 hours. In the two-hour as well as in the four-hour-groups, 3 rabbits, 1 in each endocrine state, were always inoculated with the same bacterial suspension. Rabbits to be killed at the different time intervals were not always inoculated with the same suspension. The number of *Esch. coli* in the inoculum per uterine horn was usually slightly over 200 million cells and was held as constant as possible from day to day by preparing suspensions of identical turbidity from 24-hour cultures. The numbers of *Esch. coli* and leukocytes flushed from each inoculated uterine horn were determined.

All of the ovariectomized rabbits represented in this study were ovariectomized 15 days before use.

Results and Discussion.—Rabbits in the three endocrine states were compared with regard to relative degrees of leukocytic infiltration of the endometria, leukocytic influx into the uterine lumina, and uterine

bactericidal activity (table 2). Examples of the absolute numbers of *Esch. coli* and leukocytes flushed from uteri at the various time intervals can be found in table 1 and in previous reports.[3,5]

There was little variation in the leukocytic infiltration of uninoculated uteri of rabbits in the three endocrine states. Polymorphonuclear infiltration was scant; most of the leukocytes were mononuclear. Taking into account the relative sizes of uteri, endometria of the ovariectomized rabbits probably had the least total infiltration, with no apparent difference between estrous and pseudopregnant rabbits.

Leukocytic response to uterine inoculation was fastest in estrous rabbits. Two hours after inoculation, polymorphonuclear infiltration of uteri was heavy in estrous rabbits and relatively slight in ovariectomized and pseudopregnant rabbits. The number of leukocytes in the uterine lumina was small, being only slightly greater in estrous rabbits than in the others. Slight bactericidal activity was apparent in uteri of estrous and ovariectomized rabbits at two hours, but not in pseudopregnant rabbits.

Among rabbits of all three endocrine states, there were differences in the leukocytic response by four hours postinoculation. In estrous rabbits, endometria were infiltrated more heavily than at two hours and large numbers of leukocytes had entered the uterine lumina. The response in ovariectomized rabbits was similar, but less intense. In pseudopregnant rabbits, a heavy polymorphonuclear infiltration of the endometria had occurred, but leukocytic migration into the uterine lumina lagged behind that in the other two kinds of rabbits.

The ovariectomized rabbits treated with estrogen or progesterone for the first experiment and killed four hours postinoculation showed responses typical of estrous and pseudopregnant rabbits, respectively.

The leukocytic response was noticeably different in ovariectomized and progesterone-influenced rabbits. The response in rabbits of each type was slight at two hours, possibly being somewhat greater in the progesterone rabbits; by four hours many more leukocytes were present in endometria of the progesterone-influenced rabbits. Despite this, fewer leukocytes had entered the uterine lumina of the progesterone-influenced rabbits. It seems likely that the action of progesterone either delayed the leukocytic response in the two- to four-hour period or delayed leukocytic migration through the endometrial tissue.

In rabbits of each endocrine state, the influx of polymorphonuclear leukocytes into the uterine lumina was associated with powerful bactericidal activity. This occurred between two and four hours in the estrogen-influenced and ovariectomized rabbits and between four and 16 hours in pseudopregnant rabbits.[5]

At later stages of induced infection, uteri of animals in the luteal phase contain greater amounts of pus than do uteri of animals in the follicular phase.[1,5,6]

Factors other than a difference in normal leukocytic infiltration of the endometria appear to be responsible for the differential leukocytic response in estrous and pseudopregnant rabbits. There was no discernible

TABLE 2—A Comparison Among Estrous, Ovariectomized, and Pseudopregnant Rabbits, Concerning the Migration of Polymorphonuclear Leukocytes into the Uterine Endometria and Lumina Before and During Induced Uterine Infection, with Comparative Bactericidal Activity of the Uteri

Item	Time interval post-inoculation	Type of rabbit		
		Estrous	Ovariectomized	Pseudopregnant
Endometrial infiltration	0*	±**	±	±
	2	++	+	+
	4	++++	++	+++
Luminal No.	0	−	−	−
	2	+	±	±
	4	++++	+++	+
Bactericidal activity	2	+	+	−
	4	++++	++++	+

* The number of rabbits observed at each time interval ranged from 5 to 12 in each endocrine state.
** The symbols + and − denote the degrees of leukocytic migration into the uterine endometrium and lumen, or the degree of bactericidal activity which had occurred in the uterus; −, nondetectable; ±, very limited; +, slight; ++, moderate; +++, heavy; ++++, extremely heavy.

difference in the normal infiltration of uteri in the two kinds of rabbits. Actually, the number of polymorphonuclear leukocytes in uninoculated uteri was only a small fraction of the numbers which enter the uterine lumina during the period of greatest bactericidal activity. Intense infiltration of the endometria occurred in both estrous and pseudopregnant rabbits before leukocytes entered the uterine lumina in effective numbers.

An effect on the production or release of leukotoxic substances may be the basis for the hormonal influence on the leukocytic response. Various other factors might also be affected by ovarian hormones to influence the ability of the uterus to respond to foreign substances.

SUMMARY

A study was made of the effect of ovarian hormones on the uterine defense mechanism during the early stages of induced infection.

Progesterone inhibited the bactericidal activity of the uterus against *Escherichia coli*. Estrogen stimulated the clearance of *Esch. coli* cells from the uterine lumen. The ovarian hormones apparently influenced the uterine defenses through an effect on the leukocytic response. Progesterone inhibited the response and estrogen stimulated it.

The effects of the hormones on the leukocytic response could not be accounted for by variation in the normal leukocytic infiltration of the endometria.

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SUMMARIO IN INTERLINGUA

Le Effecto de Hormones Ovarian Super le Mechanismo del Defensa Uterin Durante le Stadios Precoce de Infection Experimental

Esseva effectuate un studio del influentia de hormones ovarian super le mechanismo del defensa uterin durante le stadios precoce de infection experimental.

Progesterona inhibiva le activitate bactericida del utero contra *Escherichia coli*. Estrogeno stimulava le elimination de cellulas de *Esch. coli* ab le passage uterin. Il pare que le hormones ovarian exerceva lor influentia super le defensas uterin via un effecto super le responsa leucocytic. Progesterona inhibiva iste responsa, e estrogeno stimulava lo.

Le effectos del hormones in le responsa leucocytic non esseva explicabile per variationes in le normal infiltration leucocytic del endometrios.

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The Bactericidal Properties of Uteri and Uterine Exudates of Rabbits with Reduced Leukocytic Activity

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UTERI of estrous rabbits reduce the number of live bacteria in the uterine lumen rapidly during the first few hours of induced infection.[1,3] Uteri of rabbits in the state of pseudopregnancy require a longer time for the uterine defense to become effective.[4] The bactericidal activity of uteri in each type of rabbit is associated with the development of the inflammatory response, which occurs more rapidly in the estrous animals.[1,4]

Phagocytosis is a factor in the uterine defenses of rabbits in each endocrine state, and noncellular bactericidal substances also appear to have some defensive value to the uterus.[4] The acquisition of bactericidal properties by cell-free inflammatory exudates occurs concurrently with the influx of leukocytes into the uterine lumen,[1,3] and at least a part of these factors appear to originate in the leukocytes.[2]

Despite the association between the cellular response to introduced bacteria and bactericidal action in the uterus, the possibility has not been excluded that defensive mechanisms other than those associated with leukocytic influx are partly responsible for the endocrine-influenced variation in bactericidal activity during the early hours of induced infection. Recently, Winter *et al.*[5] found that the differential bactericidal activity of estrous and pseudopregnant rabbit uteri was similar in rabbits with induced leukopenia to the difference between untreated rabbits of the two endocrine states. Their results indicated that noncellular bactericidal factors not associated with the leukocytic response might have been active in uteri of the follicular-phase rabbits. Although the accumulation of noncellular bactericidal factors in uterine exudates of both estrous and pseudopregnant rabbits has been characteristically associated with the leukocytic response, it remains a possibility that a part of these factors are associated only in time of appearance with the leukocytes and are not a direct consequence of the leukocytic influx. Thus, some of these factors might be identical to those suggested by Winter *et al.*[5] as being active in uteri of leukopenic follicular-phase rabbits; indeed, any such factors in leukopenic rabbits might show up as powerful bactericidal agents when not in competition with other defensive elements for bacteria to kill.

A study was conducted to test for the presence of noncellular bactericidal substances in uterine exudates of leukopenic rabbits and to obtain further information on the bactericidal activity of the uterus when the inflammatory response was reduced experimentally.

EXPERIMENTAL PROCEDURES AND RESULTS

Experiment 1.—Uterine exudates of leukopenic rabbits were tested for bactericidal properties, and uteri and uterine exudates of the leukopenic rabbits were compared with those of untreated estrous and pseudopregnant rabbits with regard to bactericidal activity.

Five replicates were used, each replicate being comprised of 1 leukopenic estrous rabbit, 1 untreated estrous rabbit, and 1 untreated pseudopregnant rabbit. The rabbits were sexually mature virgin Dutch females 8 to 12 months of age. Estrous rabbits were in heat the day of use; pseudopregnant rabbits were used eight to ten days after ovulation was induced by the intravenous injection of chorionic gonadotrophin. Leukopenia was induced by the use of N-N′-N″-triethylenethiophosphoramide (TSPA), an alkylating agent used in cancer chemotherapy. Injections were made subcutaneously in sodium chloride solution, 5 to 15

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mg. per day, over a period of three or four days. Usually 10 mg. was given the first day, and succeeding doses were adjusted according to the rate of decline of the w.b.c. counts made daily. The total amount of TSPA given per rabbit varied between 19 and 40 mg. The mean w.b.c. count of the 5 TSPA-treated rabbits just prior to uterine inoculation was 750 per cmm. Nearly all of the circulating w.b.c. were lymphocytes.

Laparotomy was performed under sodium pentobarbital anesthesia, and each uterine horn of each rabbit in these five replicates was ligated at the cervix and tubo-uterine junction and inoculated intraluminally with 0.2 ml. of a saline solution (0.87% NaCl) suspension of *Escherichia coli*. The *Esch. coli* was a 24-hour culture washed from trypticase soy agar slants and was introduced into the uterus by puncturing the uterine wall carefully with a 27-gauge hypodermic needle.

Four hours after inoculation, the rabbits were killed and one horn of each rabbit flushed aseptically with 10 ml. of sterile sodium chloride solution. The uterine flushings were diluted serially and aliquots of each dilution were plated in duplicate, mixed with violet red bile agar and incubated at 37 C. for 18 to 24 hours. The plate counts gave an estimation of the number of live *Esch. coli* in the uterus after four hours of induced infection. The number of leukocytes in the flushings was estimated by spreading several 0.01-ml. aliquots over a circular area 1.0 cm. in diameter, drying, staining with methylene blue, counting the leukocytes in two microscopic sweeps at right angles through the diameter of each area, and calculating the total number of leukocytes flushed from the uterine horn.

The other uterine horn of each rabbit was flushed with 2 ml. of sodium chloride solution, these flushings being used to test for bactericidal activity of the uterine exudates. Leukocytes were removed by centrifugation at 100 g for six minutes and the supernatant fluid centrifuged again at 2,500 g for 30 minutes to remove the remaining *Esch. coli* from the uterine flushings. The supernatant fluids from the second centrifugation, containing the uterine secretions and exudates, were inoculated with 0.2 ml. of a fresh suspension of *Esch. coli*, incubated for two hours at 37 C., and the number of live *Esch. coli* determined by dilution and plate counts. The exudates of rabbits in the first two replicates were heated at 56 C. for 30 minutes before inoculation, those from rabbits in the next three replicates were not.

Just before inoculation of each exudate, several loopfuls of the fluid were spread on trypticase soy agar slants. Little or no growth resulted, indicating that few live *Esch. coli* remained in the exudates after centrifugation, certainly not enough live cells to affect significantly the numbers of *Esch. coli* alive in the exudates at the end of the incubation period.

Specimens of tissue were taken from each uterus for histologic examination.

Sterile glassware and solutions were used throughout this work.

RESULTS

The log numbers of *Esch. coli* still alive after incubation in exudates of the leukopenic and untreated rabbits are listed (table 1), along with the log numbers of *Esch. coli* and leukocytes flushed from one uterine horn of each rabbit.

Exudates from TSPA-treated estrous rabbits showed little or no bactericidal activity *in vitro*. The mean log of the *Esch. coli* inoculum per incubation tube was 8.34 (219,000,000); the mean number alive after incubation for two hours in sodium chloride solution was 8.18 (151,000,000). In comparison, the mean number alive after incubation in exudates of TSPA-treated rabbits was 8.13 (135,000,000).

Exudates from the estrous control rabbits were quite bactericidal. The mean log number of *Esch. coli* still alive after incubation was 6.88 (7,590,000), significantly less ($P<0.01$) than the number in the leukopenic rabbit exudates. Pseudopregnant rabbit exudates at this stage of inflammation were slightly bactericidal, the mean log number of live *Esch. coli* being 7.87 (74,100,000).

The lack of bactericidal substances in the cell-free uterine exudates of the leukopenic rabbits reflected the lack of bactericidal activity in uteri of the same rabbits. The mean log number of *Esch. coli* recovered from one horn of the leukopenic estrous rabbits was 8.23 (170,000,000), from the estrous controls, 6.36 (2,290,000), and the pseudopregnant rabbits, 7.87 (74,100,000).

The numbers recovered from the leukopenic and control estrous rabbits differed significantly ($P<0.01$), and uteri of the leukopenic estrous rabbits showed less bactericidal activity by four hours than uteri of the pseudopregnant rabbits.

Leukocytic response in the leukopenic rabbits was limited. The mean log number flushed from one uterine horn was 4.10 (12,600); the corresponding number from the estrous controls was 7.35 (22,400,000), and from the pseudopregnant rabbits, 6.57 (3,240,000). The relatively few leukocytes found in the flushings of the leukopenic rabbits were nearly all macrophages and were gorged with ingested *Esch. coli*. The total number of leukocytes, however, was insufficient to reduce the numbers of *Esch. coli* greatly. The leukocytes in uteri of the untreated rabbits were mostly polymorphonuclear.

The results of this experiment failed to indicate that noncellular bactericidal substances were present in uterine exudates of the leukopenic estrous rabbits. Rather, it was evident that the absence of a leukocytic response was associated with both a failure of the uterine exudates to acquire bactericidal properties and a lack of bactericidal activity in the uterine lumen.

data were obtained on the bactericidal activity of estrous rabbit uteri four hours postinoculation and on the bactericidal activity of both estrous and pseudopregnant rabbit uteri 16 hours after inoculation.

Four-Hour Bactericidal Activity.—Leukopenia was induced in 5 estrous rabbits by the use of N-(3-oxapentamethylene)-N′,N″-diethylenethiophosphoramide (OPSPA), the leukopenia-inducing drug used by Winter *et al.*[5] In addition, leukopenia was induced in 2 estrous rabbits with benzene and in 3 others with nitrogen mustard (N_2H). Ethyl alcohol and ether were also used to delay the migration of leukocytes into the uterine lumen, thus determining whether suppression of the leukocytic response by these agents had a different effect on uterine bactericidal activity than did a state of leukopenia subsequent to the action of the other compounds.

Ten or 15 mg. of OPSPA was injected subcutaneously in sodium chloride solution daily for five or six days. The total amount given varied between 60 and 80 mg. per rabbit. The w.b.c. count of the 5 OPSPA-treated rabbits just before inoculation was 740 per cmm., nearly all lymphocytes.

Benzene was given subcutaneously in a solution of equal parts of benzene and olive

TABLE 1—Log Numbers of Escherichia coli and Leukocytes Flushed from Uteri of Leukopenic Estrous Rabbits and Estrous and Pseudopregnant Controls Four Hours after Inoculation, and Log Numbers of Esch. coli Alive After Incubation In Vitro in Leukocyte-Free Exudates Obtained from the Second Inoculated Horn of Each Rabbit

		Type of rabbit								
		Estrous control			Estrous leukopenic**			Pseudopregnant control		
		In utero			*In utero*			*In utero*		
Replicate	Inoculum per uterine horn	*Esch. coli*	Leukocytes	*Esch. coli in vitro**	*Esch. coli*	Leukocytes	*Esch. coli in vitro**	*Esch. coli*	Leukocytes	*Esch. coli in vitro**
1	8.46	7.33	6.82	7.91	8.59	4.24	7.94	8.20	6.05	8.17
2	8.62	6.26	7.21	6.03	8.64	4.42	8.35	6.96	7.18	7.48
3	8.41	5.52	7.01	7.30	8.49	3.72	8.18	8.29	6.27	7.98
4	8.36	6.51	7.88	6.19	7.20	4.29	7.73	7.57	6.45	8.01
5	8.44	6.16	7.82	6.98	8.23	3.82	8.46	8.31	6.58	7.72
Mean	8.46	6.36	7.35	6.88	8.23	4.10	8.13	7.87	6.51	7.87

* The log number of *Esch. coli* used to inoculate each sample of exudate was 8.38, 8.31, 8.24, 8.35, and 8.42, respectively, for the five replicates. The log numbers alive after incubation in NaCl were 8.27, 8.21, 8.03, 8.12, and 8.27, respectively, for the five replicates; mean: 8.18 (151,000,000); **TSPA-treated rabbits.

Experiment 2.—Since the results of the first experiment were somewhat different than those obtained by Winter *et al.*,[5] a second experiment was done to obtain further information on the effect of inhibition of the inflammatory response on the bactericidal activity of the uterus. Additional

oil. Four milliliters of the solution was given daily for six days. The mean w.b.c. count for the 2 rabbits was 475 per cmm. at the time of uterine inoculation on the seventh day.

Nitrogen mustard was injected intravenously. Five milligrams were given initi-

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ally, followed by 1 to 5 mg. four days later. Uteri were inoculated seven days after the initial injection; the mean w.b.c. count for the 3 rabbits at the time of inoculation was 650 per cmm.

Alcohol was given to 5 estrous rabbits by stomach tube. Forty to 60 ml. of a 20 per cent ethyl alcohol solution was given two hours before inoculation and followed by sufficient doses to maintain a deep stupor until the rabbits were killed four hours after inoculation.

A state of deep ether anesthesia was maintained in 5 estrous rabbits from approximately one hour before uterine inoculation until they were killed four hours postinoculation.

The rabbits treated with OPSPA or N_2H, as well as the ones treated with TSPA in the previous and subsequent parts of this study, showed no obvious ill effects of the treatments. All appeared to be in good health at the time of inoculation. The benzene-treated rabbits refused food and were debilitated and obviously in poor health at the time of inoculation.

Laparotomy of the leukopenic and control rabbits was performed under sodium pentobarbital anesthesia. No pentobarbital was needed for the rabbits in alcoholic stupor or for those under ether anesthesia.

Only one uterine horn of each rabbit was ligated and inoculated. One horn was left uninoculated and, in the leukopenic rabbits, used to check the possibility that the impaired defenses of these rabbits had allowed an *Esch. coli* infection to develop in the uterus before inoculation, thus affecting the number of *Esch. coli* recovered after inoculation. Four hours after inoculation, the inoculated horn of each rabbit was flushed with 10 ml. of sodium chloride solution and determinations made of the number of *Esch. coli* and leukocytes in the flushings. The uninoculated horn was flushed with 5 ml. of sodium chloride solution and the flushings plated in violet red bile agar.

Specimens from each inoculated and uninoculated uterine horn were fixed for histologic examination.

Results.—The mean number of *Esch. coli* in the inoculum per uterine horn was 219 million cells. Mean log numbers of *Esch. coli* recovered from the leukopenic and control rabbits were as follows (table 2): OPSPA-treated, 8.40 (251,000,000); benzene or N_2H (combined), 8.06 (115,000,000); estrous controls, 5.50 (316,000); pseudopregnant rabbits, 7.92 (83,200,000).

The mean log number of leukocytes flushed from the OPSPA-treated rabbits was 3.85 (7,080) and from the benzene and N_2H-treated rabbits combined, 4.15 (14,100). In contrast, the mean for the estrous control rabbits was 8.24 (174,000,000). For pseudopregnant rabbits, the mean was 6.68 (4,790,000), being, as expected, well below the numbers in the estrous controls. As in the TSPA-treated rabbits, most of the leukocytes that were found in uterine flushings of the leukopenic rabbits were macrophages and were gorged with ingested *Esch. coli.*

It is unlikely that the numbers of *Esch. coli* flushed from the inoculated uterine horns of leukopenic rabbits were enhanced by any large numbers of *Esch. coli* present in the uterus prior to experimental inoculation, since the total number of *Esch. coli* flushed from the uninoculated uterine horn of each leukopenic rabbit never exceeded 780 *Esch. coli* cells.

It was evident again, as in the TSPA-treated rabbits, that a limited leukocytic response was associated with limited bactericidal activity in the uterus.

Alcohol and ether were effective only in some of the treated rabbits in inhibiting leukocytic migration into the inoculated uterine lumina, even though no differences were recorded between rabbits in degrees of alcoholic stupor or ether anesthesia.

The leukocytic response of rabbits in the alcohol or ether groups tended to be either very severe, similar to that in the estrous controls, or inhibited considerably. The 10 rabbits were therefore allotted to two groups according to the numbers of leukocytes flushed from the uterus. Five rabbits (3 in the alcohol treatment group, 2 in the ether group) had less than 10 million leukocytes in the uterine lumen (table 2). The treatments were undoubtedly a factor in limiting the leukocytic response in these rabbits. The other 5 similarly treated rabbits showed no inhibition of leukocytic response.

The mean log number of *Esch. coli* recovered from the 5 affected rabbits was 8.33 (214,000,000), and the mean log number of leukocytes was 5.15 (141,000). These figures were quite similar to those for the leu-

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TABLE 2—Log Numbers of Escherichia coli and Leukocytes Flushed from Uteri of Leukopenic, Alcohol-Treated, or Ether-Treated Estrous Rabbits and Estrous and Pseudopregnant Controls Four Hours after Inoculation*

Replicate	Inoculum per uterine horn	Type of rabbit and treatment											
		Estrous control		Estrous (OPSPA)		Estrous (benzene or N_2H)**		Estrous (alcohol)		Estrous (ether)		Pseudopregnant control	
		Esch. coli	Leukocytes	*Esch. coli*	Leukocytes	*Esch. coli*	Leukocytes	*Esch. coli*	Leukocytes	*Esch. coli*	Leukocytes	*Esch. coli*	Leukocytes
1	8.34	5.49	8.60	8.67	3.72	7.22	4.97	6.27	8.45	6.08	8.54	8.20	6.48
2	8.41	5.29	7.89	8.15	3.83	8.67	4.02	8.58	4.29	8.46	4.54	7.90	6.34
3	8.37	6.61	8.08	8.79	3.98	8.58	4.10	7.24	6.91	8.69	5.32	6.94	7.25
4	8.29	5.18	8.39	8.46	4.02	8.27	3.88	8.67	4.67	5.04	7.58	7.96	6.56
5	8.31	4.95	8.24	7.92	3.72	7.54	3.77	6.69	7.25	5.08	8.43	8.61	6.75
Mean	8.34	5.50	8.24	8.40	3.85	8.06	4.15	7.49	6.31	6.67	6.88	7.96	6.59

* Only one uterine horn of each rabbit was inoculated. ** Rabbits in replicates one and two were benzene-treated; those in replicates three, four, and five were N_2H-treated.

kopenic rabbits. From the 5 unaffected rabbits, the mean log number of *Esch. coli* was 5.83 (676,000) and the number of leukocytes 8.05 (112,000,000), these values being similar to those for the estrous control rabbits. The association between leukocytic response and bactericidal activity was highly significant statistically ($P<0.01$). These results show that uterine bactericidal activity was inhibited by delaying leukocytic migration into the uterine lumen just as well as by inhibiting the leukocytic response through induction of leukopenia.

16-Hour Bactericidal Activity.—By 16 hours postinoculation, the bactericidal activity of pseudopregnant rabbit uteri is generally marked, so the effect of leukopenia was determined on the uterine bactericidal action at this time interval for both estrous and pseudopregnant rabbits. Three estrous and 3 pseudopregnant rabbits were treated with TSPA. Injections of TSPA into the pseudopregnant rabbits were begun on the sixth day of pseudopregnancy and the uteri were inoculated on the tenth day. The mean w.b.c. count for the estrous rabbits at the time of inoculation was 625 per cmm., and for the pseudopregnant rabbits, 380. Each leukopenic rabbit, along with controls of each endocrine state, was inoculated in one uterine horn and killed 16 hours later. The numbers of *Esch. coli* and leukocytes were determined in flushings from the inoculated horns, and the number of *Esch. coli* in flushings from the uninoculated ones.

Results.—The uterine defenses of leukopenic estrous and pseudopregnant rabbits appeared to be inhibited completely to this stage of inflammation (table 3). The mean log number of *Esch. coli* recovered from the leukopenic estrous rabbits was 8.66 (457,000,000), and from the leukopenic pseudopregnant rabbits, 9.75 (5,620,000,000), being greater in each type of rabbit than the numbers introduced into the uterine lumen. From the estrous controls, the number was 4.53 (33,900) and from the pseudopregnant controls, 5.62 (417,000).

Similar to the previous results in estrous rabbits four hours after inoculation, the failure of the uterine defenses by 16 hours in the leukopenic estrous and pseudopregnant rabbits was associated with a paucity of leukocytes in the uterine lumen (table 3). The leukopenic estrous rabbits had a

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mean log number of 4.84 leukocytes (69,200) and the pseudopregnant ones, 4.69 (49,000). In the control estrous rabbits, the number was 8.63 (427,000,000), and in the pseudopregnant controls, 8.70 (501,000,000). The leukocytes in the leukopenic rabbit uteri were predominently macrophages and were heavily laden with phagocytized *Esch. coli*.

The number of *Esch. coli* flushed from uninoculated uterine horns varied between one and 650 per horn, thus failing to suggest that the numbers of *Esch. coli* flushed from the inoculated horns were influenced by bacteria present before inoculation.

scopic examination of stained sections of uteri from the leukopenic rabbits failed to disclose any polymorphonuclear leukocytes in the endometria. This was true for the estrous rabbits treated with TSPA, OPSPA, benzene, or N_2H and killed four hours postinoculation, and for the TSPA-treated estrous and pseudopregnant rabbits killed 16 hours postinoculation.

Polymorphonuclear infiltration of the endometrium was heavy in most of the four-hour estrous control rabbits and 16-hour estrous and pseudopregnant controls. Endometrial infiltration was moderate in the four-hour pseudopregnant controls, al-

TABLE 3—Log Numbers of Escherichia coli and Leukocytes Flushed from Uteri of Leukopenic (TSPA-treated) and Control Estrous and Pseudopregnant Rabbits 16 Hours after Experimental Inoculation*

		Estrous rabbits				Pseudopregnant rabbits			
		Leukopenic		Controls		Leukopenic		Controls	
Replicate	Inoculum per uterine horn	*Esch. coli*	Leukocytes	*Esch. coli*	Leukocytes	*Esch. coli*	Leukocytes	*Esch. coli*	Leukocytes
1	8.40	8.66	4.94	4.34	8.50	9.75	4.84	6.07	8.75
2	8.24	8.80	4.51	5.07	8.48	9.60	4.42	5.44	8.62
3	8.40	8.52	5.06	4.17	8.90	9.89	4.81	5.35	8.74
Mean	8.35	8.66	4.84	4.53	8.63	9.75	4.69	5.62	8.70

* One uterine horn of each rabbit was inoculated.

The average number of *Esch. coli* recovered from the leukopenic pseudopregnant rabbits was over ten times as great as the number flushed from similar estrous rabbits. This difference could hardly be due to the small difference in numbers of leukocytes flushed from uteri of the two kinds of rabbits. Bacterial multiplication obviously occurred in both kinds of rabbits, but apparently to a greater degree in the pseudopregnant ones. Possibly the pseudopregnant rabbit uteri provided a more nutritive environment for bacterial multiplication over a period of 16 hours, or bactericidal or bacteriostatic factors could have been active to some degree in uteri of the leukopenic estrous rabbits.

The pH of the uterine contents of the leukopenic estrous rabbits and the estrous and pseudopregnant controls was about 7.0 or slightly alkaline, while the pH of uterine contents of leukopenic pseudopregnant rabbits, probably due to bacterial growth, was about 6.0.

Histologic Examination of Uteri.—Microthough luminal infiltration was still limited at this time interval.

In the alcohol- or ether-treated rabbits, the degree of endometrial infiltration varied considerably. The rabbits with large numbers of leukocytes in the lumen had heavy endometrial infiltration. In the rabbits affected by the alcohol or ether treatment, having relatively few leukocytes in the uterine lumen, the uterine tissue was infiltrated to a moderate degree by polymorphonuclear leukocytes by four hours postinoculation, indicating that in these rabbits the leukocytic response was not inhibited completely, but was slowed to the extent that migration into the uterine lumina was still limited. The means by which these compounds slowed the leukocytic response might have been an effect on the leukocytes themselves or, directly or indirectly, on any tissue through which leukocytes pass in moving from the circulation to the uterine lumen.

Although polymorphonuclear leukocytes were absent from uteri of the leukopenic

rabbits, variable numbers of mononuclear cells were present in the endometrium. The total number of the latter was always far less than the number of polymorphonuclear leukocytes in the control rabbit endometria. Most of the former were located just beneath the endometrial epithelium.

Discussion

Uteri of the rabbits used in this study did not possess an effective defense against *Esch. coli* after the leukocytic response had been inhibited experimentally, and there was little or no evidence that bactericidal factors independent of those associated with leukocytic influx were present in the uterine exudates of leukopenic estrous rabbits. These results are somewhat different than those of Winter *et al.*,[5] who found that the differential bactericidal activity of uteri in estrous and pseudopregnant rabbits was maintained in leukopenic rabbits of the two endocrine states. Their results suggested that uteri of leukopenic estrous rabbits possessed a noncellular uterine defense mechanism. In comparing results of the two studies, however, it should be noted that experimental procedures differed somewhat. The rabbits used by Winter *et al.*[5] were given 160 to 190 mg. of OPSPA in one injection and consequently the leukocytic numbers were reduced more rapidly in their rabbits and the uteri were inoculated sooner after administration of the drug. Perhaps the more chronic action of the leukopenia-inducing compounds used in this study inhibited some extraleukocytic uterine defense mechanism which was still operative in the rabbits of Winter *et al.*,[5] or there may have been more residual leukocytes in their rabbits. In this study, there were exceptions to the rule that absence of the leukocytic response was associated with a lack of bactericidal activity in the uterus —the exceptions were the TSPA-treated rabbit in replicate four (table 1), the benzene-treated rabbit in replicate one (table 2), and the N_2H-treated rabbit in replicate five (table 2). The number of live *Esch. coli* cells flushed from a uterine horn of each of these rabbits was relatively small, but the total number of cells, live and killed, was approximately equal to the number introduced. Thus it seems possible that some noncellular bactericidal agent was active in the uteri of these rabbits.

If the leukopenia-inducing compounds used in this experiment actually did inhibit some uterine defense not associated with the presence of leukocytes, it seems likely that any such defensive mechanism was also inhibited by the alcohol or ether treatments, since in rabbits treated with these agents there was no apparent uterine defense unless leukocytes entered the uterine lumen in effective numbers.

Summary

A test was made of the bactericidal activity of rabbit uteri and uterine exudates after the leukocytic response to introduced bacteria had been inhibited experimentally.

Cell-free uterine exudates obtained four hours after experimental inoculation of leukopenic estrous rabbits had little or no bactericidal activity *in vitro*, thus failing to demonstrate the presence of noncellular antibacterial factors, independent of leukocytes, in uteri of estrous rabbits. Exudates from control estrous rabbits were highly bactericidal.

Uteri of leukopenic rabbits did not show an effective uterine defense against *Escherichia coli* by either four or 16 hours after inoculation, and uteri of leukopenic pseudopregnant rabbits showed no evidence of uterine defense by 16 hours. In contrast, uteri of estrous controls contained relatively few of the introduced bacteria by four hours and uteri of the pseudopregnant controls contained few by 16 hours. Cellular response in the leukopenic rabbits was limited to small numbers of mononuclear cells; the leukocytic response, mostly polymorphonuclear, was heavy in the control rabbits. Leukopenia was induced by N-N′-N″-triethylenethiophosphoramide, N-(3-oxapentamethylene)-N′,N″-diethylenethiophosphoramide, benzene, or nitrogen mustard.

Leukocytic migration into the uterine lumen was delayed in other estrous rabbits kept in an alcoholic stupor or under continuous ether anesthesia. In rabbits in which the treatment delayed the leukocytic influx, no bactericidal activity was apparent in the uterus.

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SUMMARIO IN INTERLINGUA

Le Proprietates Bactericida de Uteros e de Exsudatos Uterin ab Conilios con Reducite Activitate Leucocytic

Esseva testate le activitate bactericida de uteros e exsudatos uterin de conilio post que le responsa leucocytic al introduction de bacterios habeva essite supprimite experimentalmente.

Exsudatos uterin sin cellulas, obtenite quatro horas post le inoculation experimental de leucopenic conilios estrual, habeva pauc o nulle activitate bactericida in vitro, demonstrante assi le absentia, in uteros de conilios estrual, de non-cellular factores bactericida non dependente de leucocytos. Exsudatos ab conilios estrual de controlo esseva altemente bactericida.

Le uteros de conilios leucopenic non manifestava un efficace defensa contra *Escherichia coli*, tanto al fin de quatro como etiam al fin de 16 horas post le inoculation, e le uteros de leucopenic conilios pseudopregnante monstrava nulle signo del toto de defensa uterin al fin de horas. Del altere latere, le uteros de conilios estrual de controlo contineva relativemente paucos del bacterios introducite quatro horas previemente, e le uteros de conilios pseudopregnante de controlo contineva relativemente paucos del bacterios introducite 16 horas previemente. Le responsa cellular in conilios leucopenic esseva restringite a micre numeros de cellulas mononuclear. Le responsa leucocytic—principalmente polymorphonuclear—esseva marcate in le conilios de controlo. Le leucopenia esseva inducite per N-N′-N″- triethylenethiophosphoramida, N-(3-oxapentamethylene)-N′,N″- diethylenethiophosphoramida, benzena, o mustarda de nitrogeno.

Le migration del leucocytos a in le lumine del utero esseva retardate in altere conilios estrual que esseva mantenite in stupor alcoholic o sub continue anesthesia per ethere. In conilios in que un tractamento experimental habeva retardate le influxo de leucocytos, nulle activitate bactericida esseva apparente in le utero.

Noncellular Bactericidal Factors in Inflammatory Exudates from the Uterine and Pleural Cavities of Estrous and Pseudopregnant Rabbits

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INFLAMMATORY EXUDATES of rabbit uteri have been shown to contain antibacterial agents other than phagocytes.[3,4] The concentration of these substances was influenced by the endocrine state of the animal. Exudates obtained from estrous rabbit uteri a few hours after intrauterine inoculation with *Escherichia coli* or silica were highly bactericidal after the leukocytes had been removed. Similar exudates from pseudopregnant rabbit uteri were only slightly bactericidal.

Much, and possibly all, of the bactericidal activity of cell-free exudates from the estrous rabbits appeared to be due to substances originating in leukocytes. Extracts of leukocytes from uterine exudates possessed bactericidal properties similar to those of the exudates, and the bactericidal activity of cell-free exudates was consistently proportional to the number of leukocytes in the uterine lumen.[4] Thus, the presence of the noncellular substances in the uterine lumen is presumably a consequence of the leukocytic influx.

The leukocytic response and accumulation of noncellular factors were associated with a high degree of bactericidal activity in the uteri of estrous rabbits during the first four hours of induced infection.[3] In pseudopregnant rabbits, however, the cellular response was considerably slower, the cell-free exudates were only slightly bactericidal, and relatively large numbers of live bacteria were flushed from the uterus after the first four hours. During later stages of induced uterine inflammation, e.g., about 24 hours postinoculation, uteri of luteal-phase animals still contained greater numbers of live bacteria than did uteri of follicular-phase animals, even though they also contained more pus at this time.[1,2,6]

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The relationship between leukocytic influx and the bactericidal activity of cell-free exudates has not been established in pseudopregnant rabbits. Since noncellular bactericidal substances could undoubtedly influence the number of bacteria alive in the uterus, a persistently lower concentration of such factors in uteri of luteal-phase animals might explain the presence of greater numbers of live bacteria in uteri of these animals at the various time intervals studied, despite a progressively greater leukocytic response.

This study was undertaken to investigate some possible effects of the endocrine state of the animal on the bactericidal activity of inflammatory exudates and to relate the results to the uterine defense mechanism. The first experiment was conducted to determine whether the hormonal state of the animal influenced the relationship between the number of leukocytes in the uterine lumen and the bactericidal activity of cell-free exudates. The second experiment was performed to determine if bactericidal activity of cell-free inflammatory exudates seemed to be uniquely characteristic of the uterus or if similar bactericidal activity was also possessed by pleural cavity exudates of estrous and pseudopregnant rabbits.

EXPERIMENTAL PROCEDURES AND RESULTS

The rabbits used in this study were sexually mature Dutch females, 8 to 12 months of age. Estrous rabbits were in heat the day they were used. Pseudopregnant rabbits were used eight, nine, or ten days after ovulation was induced by mating to vasectomized bucks or by intravenous injection of chorionic gonadotrophin.

EXPERIMENT 1

Comparative Bactericidal Activity of Uterine Exudates of Estrous and Pseudopregnant Rabbits.—Inflammatory exudates of estrous and pseudopregnant rabbit uteri were studied at stages of inflammation which would give an

indication of any major difference between these two phases in the relationships among leukocytic response, concentration of noncellular bactericidal substances, and bactericidal activity of the uterus. The uterine contents were examined at two intervals postinoculation: at four hours, when the inflammatory response would be expected to be much greater in the estrous rabbits, and at 16 hours, when a severe inflammatory response would have developed in pseudopregnant rabbits and their uteri would generally contain more leukocytes than would estrous rabbit uteri.

Four replicates, using 3 estrous and 3 pseudopregnant rabbits each, were made in this experiment. Each rabbit was laparotomized under pentobarbital anesthesia, and each uterine horn ligated at the cervix and tubo-uterine junction; the cervix was ligated through an incision in the anterior vagina. The uterine horns were ligated at each end to prevent excessive drainage of the inoculum from the uterine lumen. Each horn was inoculated with 0.2 ml. of a sodium chloride solution (0.87% NaCl) suspension of a 24-hour culture of *Esch. coli* washed from trypticase soy-agar slants. The inoculum was introduced into the uterine lumen by puncturing the uterine wall carefully with a 27-gauge hypodermic needle.

One estrous and 1 pseudopregnant rabbit of each replicate were killed four hours after inoculation. The other 2 estrous and 2 pseudopregnant rabbits were killed at 16 hours. Fewer rabbits were allocated to the four-hour groups because the relative bactericidal activities of uterine exudates from estrous and pseudopregnant rabbits had been studied previously at this time interval.

The uterus was removed as soon as each rabbit was killed, and one horn was flushed aseptically with 10 ml. of sterile physiologic saline solution. These flushings were diluted serially, and duplicate 1-ml. aliquots of each dilution were mixed in sterile Petri dishes with violet red bile agar. The plates were incubated at 37 C. for 18 to 24 hours, the colonies were counted, and the total number of *Esch. coli* recovered from each uterine horn was calculated. The second uterine horn of each rabbit was flushed with 2 ml. of sodium chloride solution, and the leukocytes were removed by centrifugation at 100 g for six minutes. Since the large numbers of *Esch. coli* still alive after four hours in the uterine flushings of pseudopregnant rabbits might have caused misleading results in a test for comparative bactericidal activity of exudates, the supernatant fluid of the first centrifugation, containing the noncellular uterine exudates and secretions, was centrifuged a second time at 2,500 g for 30 minutes to remove the bacteria. The exudate from each rabbit was then heated at 56 C. for 30 minutes, inoculated with 0.2 ml. of a freshly prepared suspension of *Esch. coli*, incubated at 37 C. for two hours, and diluted serially. The numbers of live *Esch. coli* were calculated from the plate counts.

An estimate was made of the number of leukocytes recovered from one uterine horn of each rabbit. In those inoculated only four hours previously, this estimate was made by microscopic examination of the uterine flushings. Several 0.01-ml. aliquots of the well-mixed flushings were spread evenly over a circular area 1 cm. in diameter, dried, and stained with methylene blue. Each stained circle of uterine debris was sampled by microscopic sweeps along two diameters at right angles, the leukocytes were counted, and the total number flushed from each uterine horn was calculated. Similar examinations were made of the uterine flushings 16 hours postinoculation (PI), but clumping of the leukocytes made accurate counts by this method impossible. The number of leukocytes in one horn of each of these rabbits was estimated from the volume of cellular debris in calibrated centrifuge tubes after centrifugation of the flushings of horns used for exudate collection (0.1 ml. of cells $\cong$ 160,000,000 leukocytes). Thus, leukocyte numbers were estimated in the uterine horn of each rabbit used for determining *Esch. coli* numbers four hours PI and in the horn used for exudate collection at 16 hours PI. However, no more than slight differences were noticed in the degree of leukocytic response of the two horns of any rabbit.

During microscopic examination of the uterine flushings of each rabbit, an estimate was obtained of the total number of *Esch. coli*, killed and live, flushed from each horn. This estimate was made by counting the number of bacterial cells in many microscopic fields and calculating the total number flushed from the uterine horn. This number was compared to the number of live cells, as determined by plate counts, thus yielding information on the question of whether there was appreciable bactericidal but nonbacteriolytic action by noncellular elements in the uterus before the bacterial cells were cleared from the uterine lumen.

RESULTS

The log number of *Esch. coli* alive after incubation in exudate obtained from one uterine horn of each rabbit, and the log numbers of *Esch. coli* and leukocytes flushed from the other horn are tabulated (table 1).

The mean log number of *Esch. coli* used to inoculate the uterine exudates was 8.24 (174,000,000); the number of *Esch. coli* still alive after incubation for two hours in control tubes containing sodium chloride solution was 8.07 (117,000,000). In comparison, the following were the mean log numbers of *Esch. coli* alive after incubation in cell-free exudates of the indicated rabbits: estrous, four hours PI, 6.84 (6,920,000); pseudopregnant, four hours PI, 7.96 (91,200,000); estrous, 16 hours PI, 6.39 (2,450,000); and pseudopregnant, 16 hours PI, 3.44 (2,750). Exudates from the estrous rabbits were highly bactericidal at both

four and 16 hours. Pseudopregnant rabbit exudates were only slightly bactericidal at four hours but, with the development of the inflammatory response between four and 16 hours, the pseudopregnant rabbit exudates acquired powerful bactericidal properties. The numbers of *Esch. coli* alive after incubation in pseudopregnant rabbit exudates (obtained 16 hours PI) were significantly less than the numbers alive in estrous rabbit exudates ($P<0.01$).

As would be expected from previous experimental results,[3] the leukocytic response was slower initially in the pseudopregnant rabbits, but within 16 hours there were greater numbers of leukocytes in the uterine lumina of pseudopregnant rabbits than in those of estrous rabbits. Mean log numbers of leukocytes flushed from one uterine horn of each rabbit in the four groups were: estrous, four hours PI, 7.81 (64,600,000); pseudopregnant, four hours PI, 6.57 (3,720,000); estrous, 16 hours PI, 8.39 (245,000,000); and pseudopregnant, 16 hours PI, 8.71 (513,000,000).

No major differences were observed between estrous and pseudopregnant rabbits in the relationship between the degree of leukocytic response and the bactericidal activity of cell-free exudates. The bactericidal activity of exudates was at least grossly proportional to the number of leukocytes in the uterine lumen regardless of the endocrine state of the rabbit.

The exudates used in this experiment were heated at 56 C. for 30 minutes to ascertain that bactericidal activity was not due to thermolabile blood serum bactericidins which kill *Esch. coli*, but, rather, was due to the more thermostable substances related to leukocytic influx. However, exudates taken from other estrous rabbits at four hours and from both estrous and pseudopregnant rabbits at 16 hours were compared for the effect of heating on bactericidal activity. Heating at 56 C. for 30 minutes decreased the bactericidal activity of all exudates slightly, as reported previously,[4] but no differences in this respect were found between groups of rabbits. This failed to indicate that differential amounts of any blood serum bactericidins were present in exudates of rabbits of the different groups.

Pseudopregnant rabbit uteri contained greater numbers of live *Esch. coli* than did uteri of estrous rabbits both four and 16 hours PI. The difference between the two kinds of rabbits was considerably greater at four than at 16 hours. Mean log numbers of *Esch. coli* flushed from one uterine horn of each rabbit in the four groups were as follows: estrous, 4 hours PI, 6.07 (1,170,000); pseudopregnant, 4 hours PI, 8.22 (166,000,000); estrous, 16 hours PI, 4.66

TABLE 1—Log Numbers of Escherichia coli and Leukocytes Flushed from One Inoculated Uterine Horn of Estrous and Pseudopregnant Rabbits, and Log Numbers of Escherichia coli Alive After In Vitro Incubation in Leukocyte-Free Inflammatory Exudates Obtained from the Second Inoculated Uterine Horn of Each Rabbit

	Estrous rabbits				Pseudopregnant rabbits			
		In utero				*In utero*		
Replicate*	Rabbit No.	*Esch. coli*	Leukocytes	*Esch. coli in vitro***	Rabbit No.	*Esch. coli*	Leukocytes	*Esch. coli in vitro***
4 HOURS POSTINOCULATION								
1	1	6.31	7.33	7.43	2	8.54	5.91	8.11
2	7	6.17	7.58	7.94	8	7.76	7.05	7.77
3	13	5.78	8.14	6.97	14	8.59	6.53	8.01
4	19	6.02	8.20	5.71	20	8.01	6.79	7.93
Mean	....	6.07	7.81	6.84	....	8.22	6.57	7.96
16 HOURS POSTINOCULATION								
1	3	4.80	8.39	7.38	4	6.74	8.82	6.38
	5	3.99	8.58	7.23	6	7.44	8.47	4.04
2	9	3.65	7.92	6.12	10	6.21	8.84	5.76
	11	4.39	8.36	6.39	12	5.35	8.69	3.62
3	15	4.79	8.83	4.88	16	5.58	8.71	3.28
	17	4.22	8.47	6.09	18	5.46	8.82	0.70
4	21	5.80	8.60	5.92	22	5.97	8.85	1.81
	23	5.65	7.99	7.11	24	7.34	8.48	1.90
Mean	....	4.66	8.39	6.39	....	6.26	8.71	3.44

* Rabbits in the 16-hour replicates were inoculated at the same time and with the same inoculum as the rabbits in the corresponding four-hour replicates. The log numbers of *Esch. coli* used to inoculate each uterine horn were 8.39, 8.42, 8.30, and 8.37, respectively, for the four replicates.

** The log numbers of *Esch. coli* used to inoculate each sample of exudate were 8.35, 8.20, 8.15, and 8.24, respectively, for the four replicates. The log numbers of *Esch. coli* alive after incubation in sodium chloride solution were 8.13, 8.06, 8.01, and 8.09, respectively, for the four replicates; mean: 8.07 (117,000,000).

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(45,700); and pseudopregnant, 16 hours PI, 6.26 (1,820,000).

The difference in numbers of *Esch. coli* flushed from uteri of pseudopregnant rabbits at four and 16 hours PI indicated that the development of the inflammatory response was associated with a degree of bactericidal activity comparable to that which occurred in estrous rabbits prior to four hours. Although bacterial multiplication may have been a complicating factor, the comparative numbers of live *Esch. coli* flushed from uteri of estrous and pseudopregnant rabbits at 16 hours suggest that the pseudopregnant rabbits may have had a less effective uterine defense to this stage of inflammation, despite a greater leukocytic response. If this is so, the difference in effectiveness of the uterine defenses of the two kinds of rabbits was not due to a relative lack of noncellular bactericidal substances in uteri of the pseudopregnant rabbits.

EXPERIMENT 2

Bactericidal Activity of Pleural Cavity Exudates.—Eight estrous and 7 pseudopregnant rabbits were each inoculated in the pleural cavity with 10 ml. of a sodium chloride solution suspension of *Esch. coli;* 5 ml. was injected between the ribs on each side. The needle was inserted just through the body wall to prevent deposition of the inoculum in the lung tissue. The mean number of *Esch. coli* per inoculum was slightly over 10 billion cells (table 2).

The first 4 estrous and 3 pseudopregnant rabbits were killed six hours after inoculation. Four other estrous and 4 pseudopregnant rabbits were killed four hours PI. The skin was removed from the thoracic area and the pleural cavity was opened aseptically on each side. The exudate was removed with a pipette, and the leukocytes were removed by centrifugation at 100 g for six minutes. The volume of fluid exudate averaged about 4 ml., varying between 2 and 9 ml. per rabbit.

Unlike *Esch. coli*-induced uterine exudates, which were very seldom sanguineous, the pleural cavity exudates were often slightly bloody, and the supernatant fluid after centrifugation was often somewhat serum-colored. No differences were observed between estrous and pseudopregnant rabbits in these respects. Blood serum bactericidins would undoubtedly contribute to any *in vitro* bactericidal activity of the exudates. Therefore, each supernatant fluid was heated at 56 C. for 30 minutes to inactivate the blood serum bactericidins which kill *Esch. coli*, thus allowing the detection of more thermostable bactericidal factors similar to those which accumulate in the uterine lumen with the influx of leukocytes. This treatment killed the few live *Esch. coli* in the exudates, also.

Each sample of cell-free exudate was then inoculated with 0.2 ml. of fresh *Esch. coli* suspension and incubated at 37 C. for two hours. Equal volumes of sodium chloride solution were also inoculated and incubated. After the *in vitro* incubation period, the number of live *Esch. coli* was determined by serial dilution and plate counts.

To determine whether any bactericidal activity of the exudates was associated with appreciable lysis of the cells, an estimate was made microscopically of the total number of cells in each exudate after the incubation period.

RESULTS

The pleural cavity inflammatory exudates were highly bactericidal to the *Esch. coli* test organisms (table 2). The mean log number of *Esch. coli* used to inoculate the exudates and sodium chloride solution controls was 8.28 (191,000,000), and the mean number alive after incubation in sodium chloride solution was 8.19 (155,000,000). By comparison, the mean number alive after incubation in exudates of the 8 estrous rabbits was 5.23 (170,000) and of the 7 pseudopregnant rabbits, 5.46 (288,000). The difference between the numbers alive in sodium chloride solution and in exudates of each type of rabbit was significant statistically ($P<0.01$). The presence of relatively thermostable bactericidal substances in inflammatory exudates was obviously not a characteristic of the uterus only.

The mean log number of *Esch. coli* alive after incubation in the 4 estrous rabbit exudates obtained six hours PI was 4.67 (46,800) and in the 3 pseudopregnant rabbit exudates, 4.78 (60,300); for the four-hour exudates, the figures were 5.79 (617,000) for estrous and 5.97 (933,000) for pseudopregnant rabbits. These figures do not suggest that the endocrine state of the rabbit influenced the bactericidal activity of pleural cavity exudates, at least at the time intervals studied. This is in contrast to the marked effect of the ovarian condition on the bactericidal activity of uterine exudates, but it is in accord with the failure to find an effect of the ovarian status on the bactericidal activity of the pleural cavity.[5]

The bactericidal factors in the pleural exudates were not noticeably bacteriolytic, being similar in this respect, as well as in thermostability, to the bactericidal substances in uterine exudates.

The volume of cellular debris after centrifugation of the exudates averaged 0.10

TABLE 2—Log Numbers of Escherichia coli Alive After In Vitro Incubation for Two Hours in Leukocyte-Free Inflammatory Exudates from the Pleural Cavities of Estrous and Pseudopregnant Rabbits

Replicate	Pleural cavity inoculum	*In vitro* incubation tube inoculum	*In vitro* incubation medium*		
			Estrous rabbit exudate	Pseudopregnant rabbit exudate	Sodium chloride solution
6 HOURS POSTINOCULATION					
1	10.13	8.38	2.85	.....	8.27
2	9.93	8.28	5.77	4.27	8.19
3	10.01	8.34	4.32	5.05	8.22
4	10.00	8.35	5.75	5.02	8.28
Mean	10.02	8.34	4.67	4.78	8.24
4 HOURS POSTINOCULATION					
5	10.05	8.28	3.52	8.02	8.18
6	10.08	8.05	7.17	3.78	7.96
7	10.07	8.26	5.63	5.16	8.22
8	10.07	8.30	6.85	6.91	8.21
Mean	10.07	8.22	5.79	5.97	8.14
Over-all mean	10.04	8.28	5.23	5.46	8.19

*Each exudate was heated at 56 C. for 30 minutes before inoculation.

ml. per rabbit at six hours and 0.07 ml. at four hours (approximately 150 and 105 million leukocytes, respectively) and did not differ between estrous and pseudopregnant rabbits at either time interval. These numbers of leukocytes were undoubtedly less than the total leukocytic responses, since the pleural cavities were not washed to recover the maximum possible numbers.

Microscopic examination of cellular debris indicated that the introduced *Esch. coli* had been cleared from the pleural cavity by six hours; few free or phagocytized *Esch. coli* cells were seen. At four hours, a few *Esch. coli* cells were free in the cavity, and considerable phagocytosis was evident in some rabbits.

DISCUSSION

The results of this study indicated clearly that the presence of noncellular bactericidal substances in inflammatory exudates was not restricted to uteri of estrous rabbits. Identical or similar substances were present in exudates of pseudopregnant rabbit uteri and in pleural cavity exudates of rabbits of both endocrine states.

Uteri of the pseudopregnant rabbits in the first experiment contained consistently greater numbers of live *Esch. coli* than did uteri of the estrous rabbits at each time interval studied. The reason for this difference at four hours PI is probably explained by the differential rate of inflammatory response. The persistence of the difference at 16 hours is less easily explained, since, by this time, the pseudopregnant rabbit uteri contained both more leukocytes and a higher concentration of noncellular bactericidal factors. Actually, the mean number of *Esch. coli* alive at 16 hours, compared to the number introduced, was low in each type of rabbit uterus, thus making it difficult to assess the significance of the difference. It could be due to some basic difference in the uterine defensive mechanisms, such as a partial inhibition of the defensive elements present in pseudopregnant rabbit uteri or the action of defensive elements peculiar to estrous rabbit uteri. Such factors as differential size and shape of uterine lumina, and differential endometrial anatomy might influence the effectiveness of the uterine defenses, also. However, the explanation is quite possibly connected with the differential time lag in the inflammatory response to the inoculum, a difference which could influence in many ways the relative numbers of bacteria alive in uteri of estrous and pseudopregnant rabbits at a standard PI time interval.

In uteri of both estrous and pseudopregnant rabbits, the noncellular exudate acquires bactericidal properties concurrently with the influx of leukocytes. The presence of both the cellular and noncellular antibacterial elements is associated with bactericidal activity in the uterine lumen. Some observations were made on the roles of cellular and noncellular factors in the uterine defense mechanism by microscopic examination of uterine flushings and by making use

of the fact that intact uteri and isolated cell-free exudates differ in their manner of bactericidal action. In uteri, *Esch. coli* cells are cleared from the uterine lumen; in isolated exudates, *Esch. coli* cells are killed but not lysed.

In addition to the rabbits used in this study, observations were made on other estrous and pseudopregnant rabbits killed at various stages of uterine inflammation induced by the introduction of *Esch. coli;* the number of leukocytes and live *Esch. coli* flushed from each inoculated uterine horn was known, and the total number of *Esch. coli* cells, dead and alive, had been estimated microscopically in the uterine flushings of each rabbit.

The bactericidal action of noncellular substances was evident in uteri of many pseudopregnant rabbits. At two hours PI, the plate and microscopic counts of *Esch. coli* in the uterine flushings corresponded quite closely. By four hours, there was often a wide difference between the plate and microscopic counts; the number of live *Esch. coli* in flushings of 18 of 44 pseudopregnant rabbits was less than 25 per cent of the total number of cells estimated microscopically, indicating that most of the *Esch. coli* had been killed but were not yet phagocytized or cleared from the uterine lumen. This discrepancy between the plate and microscopic counts occurred in a higher percentage of pseudopregnant rabbits at time intervals shortly after four hours; the discrepancy decreased as the bacterial cells were generally cleared from the uterus when the inflammatory response became more severe. These observations indicated that noncellular substances often acted as defensive agents in pseudopregnant rabbit uteri, possibly being of value in holding the bacterial number in check until the inflammatory response developed to the degree necessary to eliminate the infectious agents.

Leukocytes taken from uteri of pseudopregnant rabbits during the development of the inflammatory response usually contained appreciable numbers of ingested *Esch. coli.* By 16 hours, most of the bacteria had disappeared from the uterine lumen; few bacteria could be seen free in the uterine flushings or in the phagocytes. Some exceptions were observed, since uteri of a few of these rabbits (e.g., No. 6 and 24, table 1) contained considerable numbers of live *Esch. coli;* many bacterial cells were still free in the uterine lumen, and many leukocytes were gorged with ingested *Esch. coli.* These rabbits were the ones with the fewest leukocytes in the uterine lumen at this stage of inflammation. It appears that phagocytosis, mainly, is responsible for clearance of introduced *Esch. coli* from the uterine lumina of pseudopregnant rabbits.

In estrous rabbits, the bacterial cells were cleared from the uterine lumen much sooner after inoculation and in a relatively short time interval, making it more difficult to evaluate the roles of phagocytes and noncellular factors in the uterine defenses against *Esch. coli.* At two hours postinoculation, the plate and microscopic *Esch. coli* counts were still quite high, similar to those of pseudopregnant rabbits at this stage. Between two and four hours, the *Esch. coli* disappeared rapidly from the uterus, simultaneously with the influx of large numbers of polymorphonuclear leukocytes and the acquisition of bactericidal powers by the noncellular exudates. Evidence of the action of noncellular factors was seen about two to three hours PI, but not to the extent it was seen in pseudopregnant rabbits. Appreciable numbers of phagocytized *Esch. coli* were seen during the development of the leukocytic response, but it has not been possible to determine if phagocytosis accounts for all the *Esch. coli* which disappear from the lumina of ligated horns of estrous rabbits.

It seems quite possible that the most important effect of ovarian hormones on the uterine defenses is the effect on the rate of inflammatory response. However, it cannot be concluded that the endocrine state of the animal has no other effect on the uterine defense mechanism, on either the type of defense or the antibacterial effectiveness of defensive elements.

SUMMARY

An investigation was made of some possible effects of the endocrine state of rabbits on the bactericidal activity of inflammatory exudates. Noncellular bactericidal substances, found previously in inflammatory uterine exudates of estrous rabbits and probably originating in leukocytes, were found to be present in inflammatory exudates of pseudopregnant rabbit uteri and in pleural cavity exudates of both estrous and pseudopregnant rabbits.

The bactericidal activity of cell-free uterine exudates *in vitro* was proportional to the number of leukocytes in the uterine lumen, regardless of the endocrine state of the rabbit. Four hours after experimental inoculation with *Escherichia coli*, uteri of estrous rabbits had the greater leukocytic response and more bactericidal cell-free exudates; by 16 hours, pseudopregnant rabbit uteri contained more leukocytes than estrous ones, and their exudates were the more bactericidal. Ovarian hormones apparently affected the concentration of noncellular bactericidal substances by influencing the rate of leukocytic response.

Uterine flushings of the pseudopregnant rabbits contained greater numbers of live *Esch. coli* at both four and 16 hours than did uteri of estrous rabbits; the difference at 16 hours could not have been due to a lack of noncellular bactericidal substances in uterine exudates of the pseudopregnant rabbits.

Pleural cavity exudates were highly bactericidal to *Esch. coli* after being heated to inactivate *Esch. coli*-killing blood serum bactericidins. The bactericidal activity of these exudates did not appear to be influenced by the endocrine state of the rabbits.

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SUMMARIO IN INTERLINGUA

Factores Bactericida Noncellular in Exsudatos Inflammatori ab le Cavitates Uterin e Pleural de Conilios in Estro e Pseudograviditate

Esseva interprendite un investigation de certe effectos possibile del stato endocrin de conilios super le activitate bactericida de exsudatos inflammatori. Esseva trovate que substantias bactericida non-cellular, previemente incontrate in exsudatos inflammatori del utero de conilios estrual e probabilemente de origine leucocytic, es presente in exsudatos inflammatori del utero de conilios pseudopregnante e in exsudatos del cavitate pleural de conilios si ben estrual como etiam pseudopregnante.

Le activitate bactericida de exsudatos uterin noncellular esseva, in vitro, proportional al numero de leucocytos in le lumine uterin, sin reguardo al stato endocrin del conilio. Quatro horas post le inoculation experimental de *Escherichia coli*, uteros de conilios estrual habeva plus alte numerationes leucocytic e exsudatos noncellular plus fortemente bactericida que uteros de conilios pseudopregnante. Post 16 horas, le uteros de conilios pseudopregnante contineva plus leucocytos que le uteros de conilios estrual, e lor exsudatos esseva plus bactericida. Hormones ovarian afficeva apparentemente le concentration del substantias bactericida noncellular per influentiar le mesura del responsa leucocytic.

Ablutiones intra-uterin de conilios pseudopregnante contineva plus grande numeros de vive *Esch. coli* que illos de conilios in estro, tanto post quatro como etiam post 16 horas. In le secunde caso, le differentia non es explicabile per un manco de substantia bactericida noncellular in le exsudatos ab conilios pseudopregnante.

Exsudatos de cavitate pleural esseva fortemente bactericida pro *Esch. coli* post calefaction pro inactivar le bactericidinas colicida del sero sanguinee. Le activitate bactericida de iste exsudatos non pareva esser influentiate per le stato endocrin del conilios.

XUM

The Effect of an Induced State of Leukopenia on the Uterine Bactericidal Activity in Rabbits

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BACTERIA are killed more rapidly in the uteri of rabbits [1, 5] and cows [7] whose ovaries are in the follicular phase than in those whose ovaries are in the luteal phase. In rabbits, this differential response to *Escherichia coli* is observed as soon as four hours after uterine inoculation, a time at which a marked polymorphonuclear influx has occurred in the uteri of those with ovaries in the follicular phase but not in the uteri of those with ovaries in the luteal phase.[2] *Escherichia coli* infection is combatted effectively in uteri in the luteal phase only at a longer interval after infection, when the number of leukocytes in the uterus has increased.[6]

REVIEW OF LITERATURE

Sensitization, by the injection of killed *Esch. coli*, of uteri of rabbits whose ovaries are in the follicular or luteal phase enhances the uterine bactericidal action to subsequent *Esch. coli* infection, presumably by stimulating a leukocytic influx into the uterus.[5]

A heat-stable factor, possessing bactericidal properties against *Esch. coli in vitro*, is present in the cell-free portion of uterine exudates resulting from *Esch. coli* infection,[3, 4] or from the intrauterine injection of silica.[4] At a given time interval, the difference in bactericidal activity of exudates from luteal phase and follicular phase uteri appears related to the difference in number of leukocytes present in the exudates. A bactericidal factor with similar properties has also been demonstrated in washed, ground leukocytes from uterine exudates.[4]

The association of the presence of polymorphonuclear leukocytes with the ability of the uterus to overcome bacterial invasion has been interpreted as indicating the importance of leukocytes in the uterine defense against infection.

In the present experiment, the uterine bactericidal activity was studied in rabbits in which a severe leukopenia was induced rapidly with a single, large dose of N-(3-oxapentamethylene)-N′,N″-diethylenethiophosphoramide (OPSPA),* a drug used in cancer chemotherapy.

MATERIALS AND METHODS

The experiment was of factorial design. Combinations of three factors, *viz.*, hormonal state, OPSPA treatment, and uterine sensitization, were compared for their effects on the intrauterine survival of *Esch. coli*. The experiment consisted of eight individually run replicates of 4 rabbits each.

The ovaries of 2 of the rabbits in each replicate were in the follicular phase (F); those of the other 2 were in the luteal phase (L). One rabbit (T) of each pair was treated with OPSPA; the others (NT) were not. One uterine horn of each rabbit was sensitized (S) by the injection of killed *Esch. coli* before the test inoculation of live *Esch. coli* was made into all the horns. The remaining horn of each rabbit was nonsensitized (NS). Thus each replicate was comprised of four kinds of rabbits (F-T, F-NT, L-T, L-NT). The uterine horns of these rabbits fell into eight different treatment groups designated F-T-S, F-T-NS, F-NT-S, T-NT-NS, L-T-S, L-T-NS, L-NT-S, L-NT-NS.

The rabbits used in this experiment were parous females of the New Zealand White breed. Rabbits were kept in individual cages for at least three weeks to avoid uncontrolled pseudopregnancy. Such animals were regarded as having ovaries in the follicular phase. Pseudopregnant (luteal phase) animals were obtained by the intravenous injection of 100 I.U. of chorionic gonadotrophin into rabbits in the follicular phase eight days before use.

The OPSPA treatment consisted of the subcutaneous injection of the drug in sodium chloride solution in a dosage of 50 mg. per kilogram, an amount generally found lethal to rabbits within five days after treatment. The total dosage per rabbit varied from approximately 160 to 190 mg.

Forty-eight hours after treatment, one uterine horn of each rabbit of the replicate was sensitized with killed *Esch. coli*. A laparotomy with mid-

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ventral incision was made under pentobarbital and ether anesthesia. Both uterine horns were ligated with silk thread at the tubo-uterine junction, and each cervix was grasped and ligated through an incision in the anterior vagina. One horn of each rabbit was inoculated through the uterine wall with 0.3 ml. of a fairly heavy saline suspension of heat-killed *Esch. coli* (40% light transmission in a Coleman Nepho-Colorimeter using a 655-μ filter). The laparotomy incision was closed with silk thread.

The test inoculation of both horns of all animals in the replicate was made approximately 16 hours later. A laparotomy was again performed on each rabbit, and 0.2 ml. of the same saline suspension of an 18-hour slant culture of *Esch. coli*, adjusted to the desired light transmission with a Coleman Nepho-Colorimeter, was injected into both horns of each rabbit. Counts of circulating leukocytes in the OPSPA-treated rabbits were made before each laparotomy.

The results of the test inoculations were determined four hours after injection of the live organisms. The rabbits were killed, and the contents of each uterine horn flushed out as aseptically as possible with 5 ml. of sterile saline solution. Tenfold saline dilutions of the flushings and of the initial inoculum were made serially, and 0.1 ml. of every dilution was spread over the surface of each of two eosin-methylene-blue agar plates. Colony counts were made after the plates had been incubated 48 hours at 37 C.

At the time of necropsy, portions of liver, spleen, lymph node (prefemoral), and uterine horns were fixed in 10 per cent neutralized formalin. A piece of the sternum was placed in Zenker's fixative with 10 per cent acetic acid.

Heavy smears of the uterine flushings were made on microscope slides and fixed for five minutes in absolute methanol. Before examination, the smears were stained with methylene blue.

RESULTS AND DISCUSSION

The dosage of OPSPA used in this experiment quickly produced a severe state of leukopenia. At the time the intrauterine test inoculation of live bacteria was made (about 72 hours after OPSPA treatment), counts of circulating leukocytes in the blood of rabbits treated during the follicular phase ranged from 200 to 1,150 cmm. and averaged 530 cmm.; in rabbits treated during the luteal phase, the range was 250 to 5,100 cmm. and averaged 1,670 cmm. At the time of the test inoculation, the myeloid tissues of the treated animals were extremely depleted, judging from the acellularity of the sternal marrow at the time of necropsy. The influx of polymorphonuclear leukocytes into the endometrium, generally moderate to marked in the uteri of untreated rabbits, was almost or entirely absent in OPSPA-treated animals and reflected the failure of the polymorphonuclear response to the infection.

The average number of bacteria recovered from the uterine horns of OPSPA-treated rabbits was in each group greater than that from the comparable horns of untreated rabbits (table 1). Statistical analysis revealed that the over-all bacterial recoveries from the horns of OPSPA-treated rabbits were significantly higher than those from the corresponding horns of untreated rabbits ($p<0.02$). The enhanced survival of bacteria in the uteri of leukopenic rabbits is evidence of the importance of leukocytes in the normal bactericidal function of the uterus.

TABLE 1—Mean Log Number of Bacteria Recovered from Uterine Horns of Each Group (8 Replicates)

	Follicular		Luteal	
	S	NS	S	NS
OPSPA-treated	5.009	5.990	7.528	8.556
Untreated	4.405	5.299	5.980	7.713

Mean log number of bacteria initially inoculated was 8.408.
S = Sensitized; NS = nonsensitized.

It was believed, from the work of Hawk *et al.*,[5] that sensitization increased the uterine bactericidal activity by virtue of the leukocytic accumulation which it induced. Consequently, it was expected that sensitization would be less effective in the leukopenic than in the untreated rabbits. There was no suggestion of a difference in the effect of sensitization in animals in the follicular phase (mean log No. of bacteria, 5.009 vs. 5.990 for the treated, and 4.405 vs. 5.299 for the untreated). There was a trend, however, in the animals in the luteal phase (mean log No. of bacteria, 7.528 vs. 8.556 for the treated, as compared with 5.980 vs. 7.713 for the untreated), but this difference was not statistically significant.

The fact that sensitization had an appreciable effect in lowering bacterial recoveries in leukopenic rabbits is believed to be the result of the leukocyte destruction being relatively incomplete at the time of sensitization and the consequent invasion of the uterus by the remaining polymorphonuclear leukocytes. At the time of sensitization, about 48 hours after OPSPA treatment, counts of circulating leukocytes in the blood of the treated animals ranged from approximately 3,000 to 9,000 cmm.

and averaged about 5,700 cmm. That a leukocytic response was still possible at this time was indicated by a polymorphonuclear infiltration in the subserosal connective tissue of all the uterine horns (due to the irritation of the operative procedure), and the usual presence of polymorphonuclear leukocytes in smears from the flushings of the sensitized horns of the leukopenic rabbits. Since polymorphonuclear leukocytes were observed infrequently in flushings from the nonsensitized horns of the same rabbits, their presence in the uterine lumen must have been, in major part, the result of sensitization.

The more effective bactericidal activity of the uterus during the follicular phase of the reproductive cycle is attributed to the inhibitory effect of progesterone on the uterine defense mechanisms during the luteal phase,[2,7] the nature of which is not understood. It is known that, after an induced uterine infection, the influx of leukocytes into the uterine lumen occurs much more rapidly in animals during the follicular phase.[3,5] If, at four hours after intrauterine bacterial inoculation, the difference in bactericidal activity between the uteri of rabbits in the follicular and those of rabbits in the luteal phase was due to the difference in the leukocyte content of the uterine horns, it would be expected that such a difference would disappear in the absence of leukocytes. A significant difference in bacterial recoveries ($p<0.025$) still existed, however, between the nonsensitized uterine horns of OPSPA-treated animals in the follicular (5.990) and luteal phases (8.556), despite the lack of a polymorphonuclear response to the induced infection. In view of this, it would appear that a noncellular factor, unaffected at the time when leukocytes had been destroyed by the intensive OPSPA treatment, was responsible for the relatively effective bactericidal activity which occurred in the uteri of leukopenic rabbits in the follicular phase. A factor of this kind may provide a means of bacterial destruction additional to that of polymorphonuclear leukocytes in the uteri of normal rabbits in the follicular phase.

However, the possibility cannot be excluded that some portion of the bacterial destruction in the uteri of leukopenic rabbits in the follicular phase was due to polymorphonuclear or mononuclear leukocytes. A few polymorphonuclear leukocytes sometimes were found in the endometria of OPSPA-treated rabbits, more often in uteri of those in the follicular phase. The numbers of such cells in the uterine exudates were not determined quantitatively nor could their effect on the bacterial infection be evaluated clearly.

The role of mononuclear leukocytes in the uterine bactericidal activity is unclear, also. Despite the marked destruction of lymphoid tissue in the lymph nodes and spleen resulting from OPSPA treatment, considerable numbers of lymphocytes were usually found in the endometria of leukopenic as well as in control rabbits in the follicular phase. Fewer or no such cells were observed in the endometria of rabbits in the luteal phase. The presence of these cells may have been associated with the superior bactericidal properties of uteri in the follicular phase.

SUMMARY

Severe leukopenia was induced in rabbits during the follicular and luteal phases by one injection of the drug N-(3-oxapentamethylene)-N′,N″-diethylenethiophosphoramide (OPSPA). The uterine horns of OPSPA-treated and control rabbits were inoculated with live *Escherichia coli* 16 hours after one horn of each rabbit had been sensitized by inoculation with killed *Esch. coli*.

Four hours after inoculation, significantly greater numbers of live bacteria were recovered from uterine horns of OPSPA-treated rabbits than from the corresponding control horns, indicating the importance of leukocytes in the normal bactericidal function of the uterus.

The OPSPA treatment did not have a significant effect on the response of the uteri of rabbits in the follicular or luteal phase to sensitization with killed *Esch. coli*. This was believed to be the result of the incomplete leukocyte destruction in the OPSPA-treated rabbits at the time of sensitization, permitting some degree of leukocyte infiltration into the sensitized horns.

A significant difference in the bacterial recoveries from the nonsensitized horns of leukopenic rabbits occurred with the phase of the reproductive cycle. A noncellular factor was proposed to account for the bactericidal activity in the uteri of leukopenic rabbits in the follicular phase, although some effect of residual leukocytes could not be excluded.

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Summario in Interlingua

Le Effecto Exercite per Un Inducite Stato de Leucopenia Super le Activitate Bactericida Uterin in Conilios

Grados sever de leucopenia esseva inducite in conilios durante le phases follicular e luteal per unic injectiones del droga N-(-oxapentamethylene)-N′, N″-diethylenethiophosphoramida (OPSPA). Le cornos uterin de conilios tractate con OPSPA e de conilios de controlo esseva inoculate con vive *Escherichia coli* 16 horas post que un del cornos in omne animal habeva essite sensibilisate per le inoculation de morte *Esch. coli.*

Quatro horas post le inoculation, significativemente plus grande numeros de bacterios vive esseva retrovate in le cornos uterin de conilios tractate con OPSPA que in illos del conolios de controlo. Isto indica le importantia de leucocytos in le normal function bactericida del utero.

Le tractamente con OPSPA non habeva un effecto significative super le responsa del uteros de conilios in le phase follicular o luteal a sensibilisation per *Esch. coli* morte. Isto esseva interpretate como resultato del incomplete destruction de leucocytos in le conilios tractate con OPSPA al tempore del sensibilisation e del assi permittite leve grados de infiltration de leucocytos in le sensibilisate cornos.

Significative differentias del recuperation de bacterios ab le non-sensibilisate cornos de conilios leucopenic esseva constatate inter le phases del cyclo reproductive. Es proponite le postulato de un factor non-cellular pro explicar le activitate bactericida in uteros de conilios leucopenic durante le phase follicular, ben que un certe effecto de un residuo de leucocytos non pote esser excludite.

Variations in Uterine Response to Experimental Infection Due to the Hormonal State of the Ovaries. I. The Role of Cervical Drainage, Leukocyte Numbers, and Noncellular Factors in Uterine Bactericidal Activity

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THE RESPONSE of the uterus to experimental infection, in rabbits and cows, has been found to vary with the hormonal state of the animal. Infections resulting from the inoculation of bacteria [1,2,11] or bacterially contaminated semen [3,10,11] are overcome much more rapidly in animals whose ovaries are in the follicular phase of the estrous cycle than in those whose ovaries are in the luteal phase. Response of spayed animals to uterine infection is similar to that of animals in the follicular phase, whereas response of progesterone-treated, spayed animals is like that of animals in the luteal phase.[2,11] It has been proposed, therefore, that the gonadal hormones are not necessary for the function of the uterine antibacterial defense mechanism but that progesterone inhibits this activity. This inhibitory effect of progesterone on bactericidal activity appears to be confined to the uterus.[7]

Four hours after the intrauterine inoculation of *Escherichia coli*, significantly fewer bacteria are found to survive in the uteri of rabbits in the follicular phase than in those of rabbits in the luteal phase, and a marked influx of leukocytes has occurred into the uterine lumina of the rabbits in the follicular phase.[5,8] Relatively few leukocytes are present in uteri in the luteal phase four hours after bacterial inoculation,[5] but by 16 hours the number has greatly increased.[9] At the latter time, the number of viable bacteria has greatly decreased, although it is still considerably higher than the number in comparable uteri in the follicular phase.[9] Leukocytes, in view of their association with an effective uterine bactericidal activity, appear to have an important role in the uterine defense against infection.

A heat-stable, noncellular substance with bactericidal properties against *Esch. coli in vitro* has been found in uterine exudates induced by the injection of killed *Esch. coli*[5] or silica.[6] A similar substance has been extracted from washed, ground leukocytes from uterine exudates.[6] It has been suggested that these possibly identical substances may have some importance as bactericidal factors *in vivo*.[6]

The present experiment was conducted as a study of the progress of uterine infection in rabbits in the follicular and luteal phases. Other variables were introduced in order to broaden the bases of comparison between rabbits in the two hormonal states. The uterine response to *Staphylococcus aureus*, as well as to *Esch. coli*, was studied. The effect of uterine ligation, previously considered unimportant in its effect on uterine reaction to infection, was re-examined. Uteri were examined at five, ten, 20, and 40 hours after the experimental infection had been induced. Efforts were made to study both the survival and distribution of the bacteria and the mobilization and effectiveness of the host defense mechanisms. The experiment was designed factorially to investigate the manner in which bacterial type, uterine ligation, and time modify the response to infection of uteri in the follicular and luteal phases.

MATERIALS AND METHODS

Uterine infection was studied in rabbits under the conditions created by all combinations of four factors: hormonal state (follicular or luteal); kind of bacterium inoculated *(Esch. coli* or *Staph. aureus)*; uterine ligation (ligated or nonligated); time between inoculation and death (5, 10, 20, or 40 hours). For each rabbit, determinations were made of the number of bacteria surviving in the lumina of the uterus, oviduct, and vagina; of the number of leukocytes in the uterine lumen, endometrium,

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and vagina; and of the degree of activity of a noncellular bactericidal factor in the uterine exudate.

An outline of the experimental design is presented (table 1). The ovaries of half of the 192 rabbits in the experiment were in the follicular phase; those of the other half were in the luteal phase. Half of the animals in each hormonal state received intrauterine inoculations of *Esch. coli;* the other half were inoculated with *Staph. aureus.* The uterine horns of half of the animals receiving each kind of bacterium were ligated before the inoculation of bacteria .was made; those of the remainder were not. Six animals in each treatment group were killed at 5, 6 at 10, 6 at 20, and 6 at 40 hours after the intrauterine inoculation of bacteria was made. In addition, 2 rabbits in each treatment group were killed immediately after the inoculation of bacteria. The experimental procedures were performed on 1 rabbit in each of the 32 different treatment subclasses before a similar replicate was begun. Within each of the six replicates, however, the order in which the treatments were carried out was determined at random. The rabbits killed immediately after uterine inoculation were included in the first two replicates. Usually, 12 rabbits were inoculated each week, 4 on each of three alternate days.

Mature, virgin, domestic rabbits, isolated at least three weeks to prevent uncontrolled pseudopregnancy, were considered to be in the follicular phase. The luteal phase (pseudopregnant) was produced by the intravenous injection of 100 I.U. of chorionic gonadotrophin into rabbits in the follicular phase a week before use.

Laparotomies were performed on rabbits anesthetized with pentobarbital and ether. When ligation was performed, both horns were ligated with silk thread at the tubo-uterine junction, as far caudally as possible. Both uterine horns of each rabbit were then inoculated with 0.2 ml. of a saline suspension of a 16- to 20-hour culture of either *Esch. coli* or a hemolytic, coagulase-producing Staphylococcus. The cultures were standardized to a light transmission of 25 to 30 per cent with a Fisher Electrophotometer using a 650-mμ filter, and a different culture was used for each rabbit. The laparotomy wound was closed with silk thread.

At the designated time after inoculation, the rabbits were killed. The abdominal cavity was opened, and clamps were applied immediately as far caudally as possible on the vagina and at both ends of the uterine horns if they had not been ligated. The contents of one uterine horn were flushed out with 10.0 ml. of sterile sodium chloride solution, and the final volume of the flushings was recorded. The other horn was flushed with 1.0 ml. of the saline solution, and one oviduct, with 2.0 ml. Five milliliters of saline solution were injected into the clamped portion of the vagina, withdrawn and reinjected several times, and finally withdrawn and placed into a test tube.

Tenfold dilutions of the initial inoculum, the oviduct flushings, the 10-ml. portion of the uterine flushings, and the vaginal washings were made serially in sterile saline solution. One-milliliter aliquots of each dilution or of a sufficient range of dilutions were transferred to sterile Petri plates and mixed with 10 to 15 ml. of suitable melted agar (eosin-methylene-blue agar for *Esch. coli;* 7.5% mannitol salt agar for *Staph. aureus*). The plates were incubated at 37 C., and colony counts were made 24 to 48 hours later.

To test the *in vitro* bactericidal activity of the cell-free portion of the uterine exudate, the 1.0-ml. portion of the uterine flushings was first centrifuged for 25 minutes at 13,500 r.p.m. in a Servall SS1 angle centrifuge. A 0.9-ml. portion of the supernatant fluid was transferred to a test tube (8-mm. diameter) to which was added 0.1 ml. of a standardized suspension of the same kind of bacterium which had originally been used to inoculate the uterus. In another tube, 0.1 ml. of the same bacterial suspension was added to 0.9 ml. of a sodium chloride solution. The exudate and control tubes were then incubated at 37 C. for two hours. Both before and after incubation, 0.1-ml. aliquots from each tube were serially diluted, and pour plates were made as described.

Other procedures carried out in this experiment, involving determinations (total and differential) of circulating leukocytes and the distribution of leukocytes in the vagina and uterus, are described by Broome *et al.*[4]

Statistical analysis was carried out by the methods of Snedecor,[12] using appropriate transformations to make the data more normally distributed when necessary. The method of unweighted means was used when subclass numbers were unequal.

RESULTS

A summary of probability values resulting from all of the analyses in this experiment is presented (table 2).

Before analyzing the effect of the treatments on the survival of bacteria in the uterine lumen, a logarithmic transformation was made, and the effect of the size of the initial inoculum on the final bacterial survival was determined. A significant positive correlation was found, and there was no heterogeneity among the subclass regressions. The mean over-all regression (0.177) was used to adjust all of the bacterial survival values to the average initial inoculum (log = 8.8018).

The values in table 3 represent the differences in bacterial survival between rabbits in the luteal and follicular phases under different conditions of uterine ligation and time. Mean values for the animals in the follicular phase were substracted from those in the luteal phase. In each instance, more bacteria survived in the uteri of animals in the luteal phase (differences were all positive), and over-all the differences

TABLE 1—Outline of the Factorial Experiment Designed to Compare Uterine Infection in Rabbits in the Follicular and Luteal Phases

Hormonal state	Bacterium	Uterine ligation	Hours between inoculation and death			
			5	10	20	40
Follicular	*Esch. coli*	Ligated	6*	6	6	6
		Nonligated	6	6	6	6
	Staph. aureus	Ligated	6	6	6	6
		Nonligated	6	6	6	6
Luteal	*Esch. coli*	Ligated	6	6	6	6
		Nonligated	6	6	6	6
	Staph. aureus	Ligated	6	6	6	6
		Nonligated	6	6	6	6

* Number of animals in each treatment group.

were of significantly less magnitude in the ligated group.

Differences in the numbers of luminal leukocytes in the uteri of rabbits in the luteal and follicular phases, as they varied with ligation and time, were listed in a comparable fashion (table 4). Values for luminal leukocytes were used as square root transformations of the leukocyte numbers obtained by hemocytometer counts (0.00002 of the actual number). More leukocytes were found in the uteri in the follicular phase at five and ten hours in the ligated horns and at five hours in the nonligated.

The relationship between numbers of surviving bacteria and numbers of leukocytes in the uterine lumina of the rabbits in each subclass was determined by means of the regression of bacterial numbers (logs) on numbers of leukocytes (square roots of 0.00002 the actual numbers present). Significant heterogeneity was found among the subclass regressions ($p<0.05$) and among those of the animals in the follicular phase when tested separately ($p<0.05$). No significant heterogeneity existed among the subclass regressions of the luteal phase group and a significant negative correlation was found ($p<0.05$).

The average differences in vaginal bacteria recovered from rabbits in the luteal and follicular phases at the various time intervals, subtracting the latter from the former, are listed (table 5). The differences at five and ten hours are small and fluctuate from positive at five hours to negative at ten hours. At 20 and 40 hours, however, the differences are definitely greater, more bacteria being recovered from rabbits in the luteal phase. The magnitude of these differences was not affected significantly by the kind of bacteria used or by uterine ligation.

Significantly more bacteria were recovered from the oviducts of animals in the luteal than of those in the follicular phase ($L - F = 0.46$), and there was no significant change in this difference with time, uterine ligation, or kind of bacteria.

Before an analysis could be performed on the results of the *in vitro* tests for bactericidal activity of cell-free uterine exu-

TABLE 2—Summary of Analysis of the Effects of Hormonal State, Bacterial Kind, Uterine Ligation, and Time on Bacterial Recoveries and on the In Vitro Bactericidal Activity of Uterine Exudates

Sources of variation	Bacterial recovery			Bacterial activity of cell-free exudates	
	Uterus	Vagina	Oviduct	Unadjusted for leukocytes	Adjusted for leukocytes
B	<0.01*	<0.01	<0.05	<0.01	<0.01
H	<0.01	<0.01	<0.05	.....	.....
L	<0.01	.....	.....	<0.01	<0.01
T	<0.01	<0.01	<0.01	<0.01	<0.01
BH	.....	.....	.....	.....	.....
BL	.....	.....	.....	<0.01	<0.05
BT	<0.05	.....	<0.05	<0.01	<0.05
HL	<0.01	.....	.....	<0.05	.....
HT	<0.01	<0.05	.....	.....	.....
LT	<0.01	.....	.....	<0.01	.....
HBL	.....	.....	.....	.....	.....
HBT	.....	.....	.....	<0.01	.....
HLT	<0.05	.....	.....	.....	.....
BLT	.....	.....	.....	.....	.....
HBLT	.....	.....	.....	.....	<0.01

B = bacteria; H = hormone; L = ligation; T = time. * Probability value; if no figure is given, probability was <0.05.

dates, it was necessary to determine the effect of the size of the initial bacterial inoculum on final recovery. After transformation of the numerical values to logarithms, regressions of final bacterial recovery on the initial inoculum were calculated separately (within treatment subclasses) on the results from tubes containing uterine exudate and those from the saline controls. Significant regressions were found in both groups (exudate tubes: $b = 0.7612$; control tubes: $b = 0.5882$). The final bacterial recovery values in the exudate and control groups were adjusted by means of the respective regressions to the initial inoculum, averaged over both groups ($\log = 7.4238$). The bactericidal activity of the exudates was then determined by subtracting the adjusted final bacterial recovery in the exudate tubes from that in the corresponding control tubes.

The average differences in bactericidal activities between exudates from rabbits in the luteal and follicular phases, as they varied with bacterial kind and time, are shown (table 6). Mean values for exudates from animals in the follicular phase were subtracted from those for animals in the luteal phase. Thus, positive values signify more bactericidal activity in exudates from animals in the luteal phase, and negative values indicate the opposite. The differences in bactericidal activity due to hormonal state were considerably smaller in exudates from rabbits inoculated with *Staph. aureus* than in those inoculated with *Esch. coli*. In the *Staph. aureus* group, there was greater bactericidal activity in the exudates from animals in the luteal phase at five and 20 hours; in the *Esch. coli* group, this occurred at ten and 40 hours.

An effort was made to determine whether the amount of bactericidal activity in the cell-free portion of the uterine exudate was correlated with the number of leukocytes in the uterine lumen. Values for luminal leukocytes were again used as square root transformation of the leukocyte numbers obtained by hemocytometer counts. The regression of bactericidal activity on the number of leukocytes in the lumen revealed a significant over-all correlation of 0.171. When broken down, a significant correlation ($r = 0.318$) was found in the *Esch. coli* exudates, but not in the *Staph. aureus* exudates ($r = -0.030$). Although the over-all heterogeneity of the regressions within subclasses was not significant, the regressions for *Esch. coli* and *Staph. aureus* exudates were considered sufficiently different (0.028 and −0.001, respectively) that the estimates for bactericidal activity were adjusted for the presence in the exudates of the same (over-all average) number of leukocytes by the separate regressions.

Except for a third order interaction (table 2), hormonal phase had no significant effect on the bactericidal activity after adjustment for leukocyte numbers.

Discussion

Fewer bacteria survived in uteri in the follicular than in the luteal phase at all the time intervals studied after uterine inoculation, whether infection had been induced with *Esch. coli* or with *Staph. aureus* (table 3). The uterine bactericidal activity was effective more rapidly after *Esch. coli* than after *Staph. aureus* infection (BT interaction, table 2), but this relationship was independent of hormonal phase because the faster response to *Esch. coli* occurred in uteri in both follicular and luteal phases.

The survival of greater numbers of bacteria in uteri in the luteal phase was explained to some extent by the differential loss of bacteria through cervical drainage, occurring normally to a greater extent during the follicular phase, when there is greater uterine motility and the cervix is possibly more relaxed. It would be expected that uterine ligation would have, therefore, a greater effect in retaining bacteria in uteri during the follicular than during the luteal phase. The smaller magnitude of the difference in bacterial survival (between uteri in the luteal and follicular phase) in ligated than in nonligated uteri at five hours (table 3) did not appear to arise because many more bacteria were lost via the cervix from nonligated uteri in the follicular phase than from nonligated uteri in the luteal phase. At the remaining time intervals, the magnitude of the phase differences between ligated and nonligated uteri varied considerably and did not appear to reflect primarily the differential effects due to cervical drainage. It might be expected that the largest proportion of the bacterial loss through the cervix in rabbits in the follicular phase would occur during the first hours after uterine inoculation.

Bacterial recoveries from the anterior vagina did not reflect the differential effect

TABLE 3—Average Differences in Log of Bacterial Survival in Uteri of Rabbits in the Luteal and Follicular Phases Under Various Conditions of Time and Ligation

Uterine ligation	Time (hours)			
	5	10	20	40
Ligated	1.13	1.95	1.73	.62
Nonligated	1.85	1.63	3.63	1.19

Follicular values are subtracted from luteal.

TABLE 5—Average Differences in Log of Bacterial Survival in the Vaginal Lumina of Rabbits in the Luteal and Follicular Phases Under Various Conditions of Time

Time (hours)			
5	10	20	40
0.24	—0.11	1.28	1.01

Follicular values are subtracted from luteal.

of cervical drainage in rabbits in the follicular and luteal phases, since no significant interaction arose between ligation and hormonal phase (table 2). In fact, the number of bacteria recovered from the vagina could not begin to account for the number apparently lost through cervical drainage in rabbits in either the follicular or luteal phase. It would appear that the majority of bacteria which passed through the cervix were killed rapidly in the cervix or anterior vagina or moved too far caudally to be included in the vaginal washings. The larger differences in vaginal recoveries between rabbits in the luteal and follicular phases at 20 and 40 hours (table 5) may have resulted from a continuing discharge of bacteria via the cervix in rabbits in the luteal phase at a time when the uterine infection in the animals in the follicular phase had been largely resolved.

The low levels of bacterial recovery from the oviduct suggest that the movement of bacteria from the uterus into the oviduct was slight. The larger numbers of bacteria in the oviducts of animals in the luteal phase may have been caused by the larger numbers in the uteri of these animals, resulting in a greater discharge into the oviducts.

The association between luminal leukocyte numbers and uterine bactericidal activity was next examined. In the ligated group of uterine horns, fewer bacteria survived at all time intervals in uteri in the follicular phase (table 3), but at 20 and 40 hours more leukocytes were found in the uteri in the luteal phase (table 4). The same thing occurred in the nonligated uteri, except that the advantage in leukocyte numbers was gained by the uteri in the luteal phase by ten hours, and the differences thereafter were even more marked than in the ligated group. This probably occurred in part because of the greater loss of leukocytes via the cervix from nonligated uteri in the follicular phase than from nonligated uteri in the luteal phase.

An apparent association (between uteri in the follicular and luteal phases) of greater luminal leukocyte numbers with superior bactericidal activity occurred only at early stages in which more leukocytes were found in uteri in the follicular phase. That this relationship was not found at the later time intervals (when more leukocytes occurred in the uteri in the luteal phase) may have been due largely to the effect of bacterial multiplication in the uteri in the luteal phase. Also, it may have occurred because of less effective bactericidal activity of leukocytes in uteri in the luteal phase or the greater activity of nonleukocytic bactericidal substances in uteri in the follicular phase.

If, due to the delay in the mobilization of the uterine defense mechanisms in the uteri in the luteal phase, bacterial multiplication occurred during the first hours after uterine inoculation, a larger bacterial population would have to be destroyed in uteri in the luteal phase than in the follicular phase. This would result, undoubtedly, in a greater

TABLE 4—Average Differences in Numbers of Luminal Uterine Leukocytes Between Rabbits in the Luteal and Follicular Phases Under Various Conditions of Time and Ligation

Uterine ligation	Time (hours)			
	5	10	20	40
Ligated	—29.57	—12.29	19.71	20.43
Nonligated	—7.40	30.59	40.67	28.24

Follicular values are subtracted from luteal. Values for leukocytes are derived by square root transformation of 0.00002 the true number.

TABLE 6—Average Differences in Log of Bactericidal Activity Exhibited by Cell-Free Uterine Exudates of Rabbits in the Luteal and Follicular Phases at Various Time Intervals and with Different Kinds of Bacteria

Kind of bacteria	Time (hours)			
	5	10	20	40
Staph. aureus	0.04	—0.10	0.34	—0.31
Esch. coli	—0.79	0.74	—0.83	1.17

Follicular values are subtracted from luteal.

over-all influx of leukocytes into the uteri in the luteal phase once the defense mechanisms became active. Thus, the occurrence of more bacteria and more leukocytes in the uteri in the luteal phase at the later time intervals could have occurred as a result of bacterial multiplication.

The regression of bacterial survival on numbers of luminal leukocytes within each subclass was considered to be the best estimate of the linear effect of the number of leukocytes on bacteria. The over-all heterogeneity of the subclass regressions of bacterial survival on leukocyte numbers shows that no uniform relationship existed among all the subclasses. This heterogeneity appeared to be localized primarily in the follicular phase, where the subclass correlations, even at the five-hour interval, were as often positive as negative. The bactericidal activity of the uteri during the follicular phase was not associated, therefore, in any uniform linear fashion with the number of leukocytes in the lumen. Although this does not contradict the apparent association of leukocyte numbers with bactericidal activity, it may indicate that nonleukocytic bactericidal factors are operative in uteri during the follicular phase also, or that the bactericidal properties of leukocytes themselves are not entirely related to the numbers present in the uterus during the follicular phase.

The significant negative correlation in the animals in the luteal phase between bacterial survival and numbers of luminal leukocytes indicates that, in this group, the amount of bacterial destruction was directly associated with the number of leukocytes present. The different bacteria-leukocyte relationships found in uteri in the follicular and luteal phases may be associated with the phase variation in uterine response to infection.

The procedure used to measure the *in vitro* bactericidal activity of cell-free uterine exudates was essentially the same as that used by Hawk,[5] except that bacteria and leukocytes were separated from the exudates by a single centrifugation.

Over the 40-hour period, no significant difference due to hormonal state existed in the amount of cell-free bactericidal activity in the uterine exudates against either *Esch. coli* or *Staph. aureus* (failure of hormone effect or BH interaction, table 2). However, considerably more bactericidal activity against *Esch. coli* was found in *Esch. coli* exudates of animals in the follicular phase at the five-hour interval (table 6), as observed by Hawk at four hours.[5] With *Staph. aureus* exudates, on the other hand, the differences at the five- and ten-hour intervals were variable and slight. That this interaction (BHT, table 2) no longer occurred after adjustment for leukocyte numbers was due largely to a diminution of the differences in bactericidal activity against *Esch. coli* which had existed over the four time intervals. This tends to support Hawk's suggestion that the phase difference in the *in vitro* bactericidal activity of cell-free uterine exudates against *Esch. coli* is related to the phase difference in numbers of luminal leukocytes.[6]

If noncellular factors have a role in the uterine defense mechanism, it is not certain to what extent the bactericidal factors detected *in vitro* are involved. If they are involved, the earlier production of the noncellular bactericidal factor in uteri in the follicular than in luteal phase after *Esch. coli* infection would help account for the more rapid bacterial destruction in uteri during the follicular phase, as suggested by Hawk.[6] Although the bactericidal factor against *Staph. aureus* might function similarly *in vivo*, its importance would appear to be considerably less.

SUMMARY

Uterine infections were induced experimentally in rabbits whose ovaries were in the follicular phase and in the luteal phase, respectively. The differences in bacterial survival in rabbits of the two hormonal states were compared at four time intervals (5, 10, 20, and 40 hours) after inoculation, under different conditions of uterine ligation, and with the use of two kinds of bacteria (*Escherichia coli* and *Staphylococcus aureus*). The bactericidal activity of cell-free uterine exudates was determined *in vitro* against the kind of bacterium used to induce the exudate.

Under all conditions, more bacteria survived in uteri in the luteal phase than in the follicular phase.

Apparently, more bacteria were lost through cervical drainage from uteri in the follicular phase than in the luteal phase, especially during the first hours after bacterial inoculation.

The number of surviving bacteria was

related linearly to the number of leukocytes in the uterine lumen in animals in the luteal phase but not in follicular phase, possibly indicating a phase difference in the activity of leukocytes or the operation of a nonleukocytic bactericidal factor in uteri during the follicular phase.

No over-all difference due to hormonal state existed in the amount of bactericidal activity exhibited by cell-free uterine exudates *in vitro*. However, at five hours after inoculation, considerably more bactericidal activity against *Esch. coli* existed in exudates of animals in the follicular phase.

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SUMMARIO IN INTERLINGUA

Variationes del Responsa Uterin a Infection Experimental, Determinate per le Stato Hormonal del Ovarios

1. Le Rolo de Drainage Cervical, Numeration Leucocytic, e Factores Noncellular in le Activitate Bactericida del Utero

Infectiones uterin esseva inducite experimentalmente in conilios con ovarios in le phase (1) follicular e (2) luteal. Differentias in le superviventia de bacterios esseva studiate per comparar le conilios in le un e le altere stato hormonal a quatro intervallos de tempore (5, 10, 20, e 40 horas) post le inoculation, sub diverse conditiones de ligation uterin, e con le uso de duo typos de bacterio *(Escherichia coli* e *Staphylococcus aureus)*. Le activitate bactericida de acellular exsudatos uterin esseva determinate in vitro contra le typo de bacterio usate in inducer le exsudato.

Sub omne conditiones, plus numerose bacterios superviveva in uteros in le phase luteal que in uteros in le phase follicular.

Il pare que plus grande quantitates de bacterios esseva perdite per drainage cervical ab uteros in le phase follicular que ab uteros in le phase luteal, specialmente durante le prime horas post le inoculation bacterial.

Le numero del supervivente bacterios esseva relationate linearmente al numero del leucocytos in le passage uterin in le caso de animales in le phase luteal sed non in le caso de animales in le phase follicular. Isto indica possibilemente un differentia inter le phases in le activitate leucocytic. Il es etiam possibile que le phenomeno es causate per le action de un factor bactericida non leucocytic que es presente in le utero durante le phase follicular.

Nulle differentia general causate per le stato hormonal existeva inter le magnitudes del activitate bactericida manifeste in acellular exsudatos uterin in vitro. Tamen, cinque horas post le inoculation, un considerabilemente plus intense activitate bactericida contra *Esch. coli* existeva in exsudatos ab animales in le phase follicular que in exsudatos ab animales in le phase luteal.

Variations in Uterine Response to Experimental Infection Due to the Hormonal State of the Ovaries. II. The Mobilization of Leukocytes and Their Importance in Uterine Bactericidal Activity

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MANY RECENT reports have indicated that the granulocytic system plays an important role in uterine defense. Differences in the bactericidal action of estrous and pseudopregnant rabbits' uteri have been ascribed to differences in leukocytic activity in the two types of uteri soon after infection.[4] However, there is little evidence to indicate the reason for this differential ability of the leukocytes to cope with infection or to show the precise mechanism by which they eliminate bacteria from the uterus.

In the present study, leukocytic mobilization in estrous (follicular) and pseudopregnant (luteal) rabbits' uteri has been studied from three aspects: (1) the availability of leukocytes in the blood supply, (2) their mobilization by the uterus, and (3) their passage into the uterine lumen from the endometrium. In addition, an investigation has been made into the role of phagocytosis in uterine defense.

MATERIALS AND METHODS

A 2 x 2 x 2 x 4 factorial experiment involved 208 sexually mature, virgin female rabbits. The uterine defense mechanism was studied in the follicular and luteal phases to observe the effects of uterine drainage (ligated and nonligated) and the duration of infection (5, 10, 20, and 40 hours). *Staphylococcus aureus* was used to infect half the animals to facilitate the study of phagocytosis (since it is a gram-positive organism); *Escherichia coli* was used to infect the others. Six animals were randomly assigned to each of eight subgroups studied at 5, 10, 20, and 40 hours, while 8 animals served as zero-hour controls for each reproductive state. The methods of preparing the animals for infection, of infecting and ligating the uteri, and of recovering the bacteria and inflammatory exudates have been described previously.[9] In addition, the length of each uterus was measured, and portions from two separate locations of the uterus and oviduct were fixed in buffered formalin-sodium chloride solution at necropsy. They were subsequently embedded in paraffin, sectioned at 7 μ, and stained with Giemsa's stain for histologic study.

Circulating leukocyte numbers were determined by hemocytometer counts on blood taken from the marginal ear vein immediately before the anesthesia preceding infection and again immediately before the rabbits were killed. Smears of blood collected in the same way were stained by the method of Wright for differential leukocyte counting.

The total number of neutrophils in the uterine lumina was estimated by hemocytometer counts on the inflammatory exudates. The volume of exudate, also, was measured and multiplied by the hemocytometer count to produce a value representing total luminal neutrophils. A similar procedure was used to estimate vaginal neutrophils.

The phagocytic activity of uterine neutrophils was determined by placing 0.1 ml. of exudate on a clean microscope slide and spreading it over exactly 1 sq. cm. These smears were air-dried before staining with the gram technique and examination under oil immersion. The total number of neutrophils, the number of neutrophils containing bacteria, and the number of bacteria within the neutrophils were counted in 20 fields on each smear.

Calculation of total endometrial neutrophils necessitated a knowledge of the number of neutrophils in a unit volume of tissue (e.g., in a microscopic field) and the total volume of tissue present. Since the concentration of neutrophils in the glandular region differed from that in the stromal region of the endometrium, they were estimated separately. This was accomplished by taking the average neutrophil count in each of 12 microscopic fields in the glandular region and in six in the stroma. These fields were located as nearly as possible in the same position in each uterine section, using the mesometrium as an orientating landmark.

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To measure the total volume of tissue in each uterus, sections from two different locations were projected (x 20) onto a white background, and the outlines of glandular and stromal regions were traced with a planimeter. Using the appropriate factor, the average planimeter readings were converted into units equivalent to the area of the microscopic field used for counting the endometrial neutrophils. This made possible the calculation of the average number of neutrophils per histologic section which, when multiplied by the length of uterus (in terms of section thickness), provided an estimate of total glandular and stromal leukocytes in each uterus. This estimate was improved by applying Abercrombie's [1] correction factor to section thickness and microscope field area.

STATISTICAL PROCEDURES

All analyses have been carried out with the methods outlined by Snedecor.[8] Before analysis, all data on uterine neutrophils were divided by 50,000 and their distribution normalized by square root transformation; percentages were made more normally distributed by angle transformations. Blood leukocyte values appeared to be normally distributed without transformation. The method of unweighted means was used to analyze data with unequal subclass numbers.

RESULTS

An over-all summary of the statistical analyses from this experiment is presented (table 1).

1) *Circulating Leukocytes.*—Polymorphonuclear leukocyte levels increased rapidly in animals in both follicular and luteal phases following uterine infections (table 2). These elevated levels appear a little less persistent in animals in the luteal phase (not statistically significant). The level of mononuclear cells falls rapidly after infection and then gradually recovers with time. The drop is more severe ($p = 0.05$ with Duncan multiple range test [2]) and the recovery is slower in animals in the luteal phase.

2) *Number of Neutrophils in Uterine Lumen.*—Before carrying out an analysis of variance on these data, the relationship between number of luminal neutrophils and number of bacteria (variability in plate counts arose because inocula were standardized only by light transmission before use) used to infect the uteri was examined. A nonsignificant correlation was found and, therefore, no adjustments of the observed data were made.

In order to simplify this discussion, the difference between animals in the luteal and follicular phases is termed "hormone response." It is represented by subtracting the mean values for the uteri of animals in the follicular phase from mean values for the uteri of animals in the luteal phase. Negative values, therefore, indicate that more neutrophils were recovered from uteri in the follicular phase, and positive values indicate the reverse.

With both bacterial infections, more neutrophils were recovered from uteri in the follicular phase five hours after infection (table 3); subsequently, however, the uteri in the luteal phase contained more neutrophils. The magnitude of the hormone response was greater in animals infected with *Esch. coli* at five and ten hours, whereas at 20 and 40 hours the response was greater

TABLE 1—Summary of Probability Values for Analyses of Variance

Source of variation	Blood leukocytes				Vaginal leukocytes	Uterine leukocytes					
	Polymorph.		Mononuclear						In lumen	Phagocytic activity	
	Initial	Final	Initial	Final		Luminal	Endometrial	Total	(%)	No. act.	Act. (%)
H	—[a]	—	—	<0.05	—	<0.01	<0.01	<0.01	<0.01	<0.01	—
B	—	—	—	—	—	<0.05	<0.01	—	—	....[c]	....
L	—	—	—	—	—	<0.01	<0.01	....[b]	A	<0.01	—
T	—	<0.01	—	<0.01	—	<0.01	—	<0.01	<0.01	<0.01	<0.01
H × B	—	—	—	—	—	—	—	<0.05	—	....	....
H × L	—	—	—	—	—	<0.01	—	....	....	—	—
H × T	—	—	—	—	—	<0.01	<0.01	<0.01	—	<0.01	—
B × L	—	—	—	—	—	—	—	....	....	....	....
B × T	—	—	—	—	—	<0.01	—	—	<0.05	....	....
L × T	—	—	—	—	—	<0.01	—	....	....	—	—
H × B × L	—	—	—	—	—	<0.01	—	....	....	....	....
H × L × T	—	—	—	—	—	—	—	....	....	—	—
H × B × T	—	—	—	—	—	<0.05	—	—	—	....	....
B × L × T	—	—	—	—	—	—	—	....	....	....	....
H × B × L × T	—	—	—	—	—	—	—	....	....	....	....

[a] $P > 0.05$ for mean squares designated by a dash (—); numerical values where given are probability values. [b] Results from ligated uteri, only, analyzed. [c] Results with *Staph. aureus*, only, analyzed. H = reproductive state; B = bacteria; L = ligation; T = time.

with *Staph. aureus* infections. These effects were consistent in both ligated and nonligated uteri. Though ligated uteri always contained more neutrophils than nonligated uteri, the hormone response was larger in the latter (table 4). The effect of ligation was greatest in uteri infected with *Esch. coli* at all time intervals.

tal number of bacteria phagocytosed by uterine neutrophils is much higher in ligated uteri in the follicular phase at five hours than in ligated uteri in the luteal phase (table 8). At subsequent time intervals, phagocytosis appears to decrease in ligated uteri in the follicular phase but increase in those in the luteal phase to 20

TABLE 2—Changes in Circulating Leukocytes* Produced by Experimental Uterine Infections

Type of leukocyte	Reproductive phase	Duration of infection (hr.)				
		0	5	10	20	40
Polymorphonuclear	Follicular	3,675	7,346	8,570	8,131	6,507
	Luteal	3,531	8,910	8,169	6,536	3,452
Mononuclear	Follicular	9,110	6,648	7,549	7,910	8,767
	Luteal	9,172	3,994	5,336	7,026	9,765

* Values represent average number cells/cmm. of blood.

3) *Endometrial Neutrophils.*—More endometrial neutrophils were present in uteri in the follicular phase than in those in the luteal phase at five hours; the reverse was true at the later time intervals (table 5). Endometria of ligated uteri always contained more neutrophils than endometria of nonligated uteri. These effects were consistent in uteri infected with either bacterial species.

4) *Vaginal and Oviduct Neutrophils.*—No effects of ligation on vaginal neutrophils were noted, but the effect of reproductive phase approached significance. There appeared to be a complete absence of neutrophils in flushings and histologic sections from the oviduct.

5) *Total Uterine Leukocytes.*—Immediately before infection, uteri in both follicular and luteal phases appear to contain the same number of neutrophils (table 6). Neutrophil numbers increased more rapidly in uteri in the follicular phase following infection, but they eventually reached much higher levels in uteri in the luteal phase.

6) *Percentage of Total Uterine Neutrophils Present in the Lumen.*—The percentage of total uterine neutrophils in the lumen at any particular time interval was always lower in animals in the luteal phase (table 7). The variations occurring with time were consistent for uteri in both reproductive phases whether infected with *Esch. coli* or *Staph. aureus.*

7) *The Phagocytic Activity of Uterine Neutrophils.*—These results are confined to uteri infected with *Staph. aureus.* The total number of bacteria phagocytosed by uterine neutrophils is much higher in ligated uteri in the follicular phase at five hours than in ligated uteri in the luteal phase (table 8). At subsequent time intervals, phagocytosis appears to decrease in ligated uteri in the follicular phase but increase in those in the luteal phase to 20 hours before declining. The percentage of active neutrophils was higher in uteri in the luteal phase at all time intervals, as was the number of bacteria per active neutrophil.

DISCUSSION

The results of this study (table 3) are in agreement with earlier reports [4] showing that more leukocytes are active in the early stages of experimental infections in uteri in the follicular phase than in uteri in the luteal phase. It has been suggested [5,6] that differences in the bactericidal action of cows' uteri in the follicular and luteal phases might be associated with differences in the number of leukocytes (particularly neutrophils) circulating in the blood of the animals. From table 2 it is apparent that, immediately prior to infection, there were only slightly more circulating neutrophils in animals in the follicular phase (not statistically significant). Within five hours after infection, animals in the luteal phase had the higher level of blood neutrophils (again not statistically significant). Therefore, it is unlikely that, during this critical period, there was ever sufficient difference in the circulating neutrophil levels between animals in the follicular and luteal phases to have any significant effect on the bactericidal action exhibited by their uteri.

Many workers have noted the more frequent occurrence of neutrophils in cattle uteri at, and about, the time of heat. Skjerven [7] has suggested the presence of these neutrophils might be related to the high degree of bactericidal activity exhibited by

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uteri in the follicular phase. The present study revealed that the concentration of neutrophils was usually greater in normal rabbit uteri during the follicular phase. However, there was more endometrial tissue in the uteri during the luteal phase so that, when calculated on the basis of total neutrophils per uterus, the neutrophil contents of uteri in both phases were almost identical (table 6). This suggests that differences in the number of neutrophils normally present in uteri in the follicular and luteal phases have very little influence on the course of uterine infections.

TABLE 3—The Effect of Time and Bacterial Species on the Hormone Response of Luminal Neutrophils* Following Uterine Infections

Bacterial species	Duration of infection (hr.)			
	5	10	20	40
Staph. aureus	−14.26	+2.2	+37.55	+35.70
Esch. coli	−22.77	+6.10	+22.81	+12.65

* Square root (total luminal neutrophils × 0.00002).

By combining those data for total endometrial neutrophils with those for total luminal neutrophils, estimates were obtained of the total number of neutrophils attracted to the various uteri (table 6). Analysis showed that, starting from the same point, uteri in the follicular phase were able to attract neutrophils faster than uteri in the luteal phase. This implies that uteri in the follicular phase are able to produce or release (or both) some leukotactic principle at an earlier stage in the infection process than uteri in the luteal phase. This conclusion is supported by Hawk's observation [4] that inflammatory exudates, taken from estrous rabbits' uteri four hours after infection, stimulated earlier than normal leukocyte mobilization in pseudopregnant uteri infected with *Esch. coli*.

TABLE 4—The Effect of Ligation and Bacterial Species on the Hormone Response of Luminal Neutrophils* Following Uterine Infections

Bacterial species	Uterine ligation	
	Ligated	Nonligated
Staph. aureus	+9.9	+20.62
Esch. coli	−10.94	+25.44

* Square root (total luminal neutrophils × 0.00002).

In addition to variations in the rate of leukotaxine production, there may be a difference, also, between uteri in the follicular and luteal phases in the ability of neutrophils to migrate through their endometria. Examination of table 6 indicates that, at every time interval, the lumen of uteri in the luteal phase contained a smaller percentage of the total uterine neutrophils than the lumen of uteri in the follicular phase. This may indicate that it is more difficult for neutrophils to enter the lumen of uteri during the luteal phase after they have been attracted from the blood system. Alternatively, it may indicate a weaker attracting force directing the neutrophils to the lumen of uteri in the luteal phase.

Turning to the effects of ligation, it was shown in table 4 that there was a greater difference in the number of neutrophils recovered from nonligated uteri in both phases than when these uteri were ligated. It is well known that conditions in the reproductive tract during the follicular phase favor the removal of uterine debris via the cervix. Thus, the cervix is comparatively relaxed at estrus, and muscular activity is increased, as is the secretion of mucus. If drainage is impeded by ligation, the greatest effect on luminal neutrophils would be expected in uteri in the follicular phase, which is in agreement with the above results. Confirmation of this could not be obtained from estimates of uterine drainage made by counting vaginal neutrophils.

The results of Winter *et al.*[9] indicate that uterine drainage also allows the escape of bacteria, particularly from uteri in the follicular phase, during the early hours of infection. It might be anticipated that any reduction in the bacterial infection would provide a smaller stimulus for leukotaxine production and, consequently, fewer neutrophils would be mobilized by nonligated uteri. Table 5 shows that there were significantly fewer neutrophils in the endometria of nonligated than ligated uteri. This provides evidence for a lowered leukocytic response in nonligated uteri but does not explain the differential effect on luminal neutrophils produced by ligating follicular and luteal uteri.

Although no effects of uterine ligation could be demonstrated by counting vaginal leukocytes, there was an almost significant effect of reproductive phase. This may indicate that there is some difference in the

ability of the vagina to mobilize leukocytes coincident with reproductive phase. Observations on flushings and histologic sections taken from the oviducts usually failed to reveal the presence of any neutrophils. Therefore, the possibility exists that the oviduct is incapable of responding to infections in the same way as the uterus.

Another important aspect of this study concerns the mechanism by which bacteria are killed and eliminated by the uterine defense system. Hawk[4] considers that a noncellular bactericidal factor arising from the uterine leukocytes may be important in the destruction of *Esch. coli*. However, he has been unable to show that this factor possesses any lytic properties *in vitro*, which suggests that, though it may be important in killing bacteria, it is incapable of removing them from the uterus. These observations have been confirmed by the results of Winter *et al.*[9] The latter also indicate that noncellular inflammatory uterine exudates possess little bactericidal activity against *Staph. aureus*. Therefore, it is unlikely that a defense system based entirely on the production of noncellular bactericidal factors would be capable of dealing with the many bacterial species which the uterus can control.

To obtain some indication of the importance of phagocytosis in uterine defense, the number of live bacteria known to have disappeared from the uteri[9] was compared with the number present in their luminal neutrophils (table 8). Bacteria equivalent to approximately 50 per cent of the live organisms used to infect ligated uteri in the follicular phase were located in their neutrophils at five hours. In uteri in the luteal phase, more bacteria were observed in the luminal neutrophils at 20 hours than had been used to infect the uteri. The pronounced drop in phagocytosed bacteria, which occurs in ligated uteri in the follicular phase between five and ten hours and in ligated uteri in the luteal phase between 20 and 40 hours, is presumed to result from bacterial digestion by uterine neutrophils. If these neutrophils are capable of killing the bacteria as well as digesting them, it appears that phagocytosis must play an important role in uterine defense.

It is possible that Hawk's noncellular bactericidal factor may control bacterial multiplication before phagocytosis is completed. No evidence has been obtained in this experiment to indicate whether the bacteria are killed before or after phagocytosis. Further studies are needed to decide whether the uterine neutrophils kill and eliminate bacteria alone or with the aid of some noncellular factor.

TABLE 5—The Effect of Time and Reproductive Phase on Endometrial Neutrophils* Following Experimental Infections

Reproductive phase	Duration of infection (hr.)			
	5	10	20	40
Follicular	9.44	6.51	7.60	6.30
Luteal	6.40	8.97	9.97	11.24

* Square root (stromal plus glandular neutrophils per histologic section).

The question now arises as to whether the differing bactericidal action of uteri in the follicular and luteal phases results entirely from the earlier mobilization of follicular leukocytes. Winter *et al.*[9] pointed out that,

TABLE 6—The Effect of Time and Reproductive Phase on Total Uterine Neutrophils* Following Infection of Ligated Uteri

Reproductive phase	Duration of infection (hr.)				
	0	5	10	20	40
Follicular	4.57	79.80	86.12	94.05	93.67
Luteal	4.56	22.49	74.06	137.05	137.12

* Square root (endometrial + luminal neutrophils × 0.00002). Response pattern was the same in uteri infected with either bacterial species.

by 20 and 40 hours after infection, there were usually more leukocytes and also more bacteria in uteri in the luteal phase than in uteri in the follicular phase. This suggests uterine leukocytes in the luteal phase are unable to deal with uterine infections as efficiently as those in the follicular phase. This is supported by the observation of Hawk *et al.*[3] that a significant difference in the bactericidal action of uteri in the luteal and follicular phases existed even after sensitization.

TABLE 7—Percentage of Total Uterine Neutrophils Present in the Lumen Following Experimental Infections

Reproductive phase	Duration of infection (hr.)			
	5	10	20	40
Follicular	86.6%	95.5%	95.2%	95.8%
Luteal	56.6%	72.9%	90.9%	89.9%

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It is possible that the slower mobilization of the defense system during the luteal phase allows invading bacteria to multiply and thus increases the severity of infection. Evidence in support of this hypothesis is presented (table 8) which shows that the number of bacteria observed in neutrophils from uteri in the luteal phase reached approximately twice the level used to infect the uteri by the 20-hour time interval. If bacterial digestion also occurred during this period, a considerable amount of bacterial growth seems to have taken place.

Another possibility is that the leukocytes per se are more effective in uteri during the follicular phase. However, it was impossible for Winter *et al.*[9] to demonstrate any difference in the production of noncellular bactericidal factors by uterine leukocytes associated with reproductive phase. Similarly, estimates of the number of bacteria per active neutrophil and the percentage active neutrophils (table 8) are no greater in uteri in the follicular phase. The data concerning the rate of bacterial digestion by neutrophils of uteri in the follicular and luteal phases are complicated by bacterial multiplication. However, if the number of bacteria disappearing from uterine neutrophils during the follicular phase between five and 20 hours is compared with those disappearing from uterine neutrophils during the luteal phase between 20 and 40 hours, very little difference can be observed.

Winter *et al.*[9] have investigated the possibility that some "nonleukocyte number" factors may be affecting the apparent efficiency of follicular and luteal leukocytes. They showed that a linear association between uterine bactericidal action and leukocyte numbers existed only in uteri in the luteal phase. The absence of a similar association in uteri in the follicular phase may indicate the operation of some nonleukocytic defense system.

In considering the factors responsible for differential survival of bacteria after uterine infections in rabbits in the follicular and luteal phases, the rate of leukocyte mobilization and passage into the lumen appear important. The more rapid influx of leukocytes into the lumina of animals in the follicular phase, possibly the result of an earlier production of a leukotactic substance, is associated with more rapid destruction of bacteria. There is also evidence that bacterial multiplication occurs in uteri in the luteal phase before the defense mechanisms are fully mobilized. This would increase and prolong the phase difference in bacterial survival and help account for the greater over-all response in uteri in the luteal phase. However, the results also suggest that a nonleukocytic defense mechanism may be operating in uteri in the follicular phase.

TABLE 8—The Effect of Time, Ligation, and Reproductive Phase on Uterine Phagocytosis

Item	Reproductive phase	Uterine ligation	Duration of infection (hr.)			
			5	10	20	40
Total No. bacteria in neutrophils [a]	Follicular	Ligated	28.8	5.0	2.5	2.07
		Nonligated	1.0	4.0	2.1	0.8
	Luteal	Ligated	1.75	38.0	106.0	45.0
		Nonligated	3.0	30.0	93.0	13.0
Active neutrophils (%)	Follicular	Combined [b]	33.65	27.46	12.87	11.27
	Luteal	Combined	61.52	60.53	42.01	28.62
No. bacteria per active neutrophil	Follicular	Combined	11.56	8.71	4.32	3.83
	Luteal	Combined	14.56	15.03	13.32	6.75

[a] Average number bacteria observed within luminal neutrophils $\times 10^{-7}$. [b] No effect of ligation; therefore, ligated and nonligated results have been combined.

Phagocytosis appears to be an important factor in the elimination of *Staph. aureus* (and possibly *Esch. coli*) from uteri in both phases. It is not certain to what extent noncellular factors, either of leukocytic origin or independent of leukocytes, function in uterine defense. Cervical drainage occurs to a greater extent in uteri during the follicular phase but does not appear to be the major reason for the phase difference in uterine bactericidal activity.

Summary

A study has been made of various aspects of leukocyte mobilization by uteri of rabbits in the follicular and luteal phases, following experimental infections with *Escherichia coli* and *Staphylococcus aureus*. The results fail to indicate that differences in the level of leukocytes circulating in the blood of animals in the different reproductive phases are responsible for the difference in bactericidal action of their uteri. Similarly, this differential bactericidal action does not arise from a difference in the number of neutrophils normally present in the uteri during the follicular and luteal phases prior to infection. Evidence is presented which suggests uteri in the follicular phase are capable of producing, or releasing, a leukotactic factor at an earlier stage of the infection process than uteri in the luteal phase. The results also suggest that it is more difficult for leukocytes to pass through the endometrium of uteri in the luteal phase once they have been mobilized from the blood stream.

Ligation has a greater effect on the number of leukocytes accumulating in uteri in the follicular than in the luteal phase. In addition to disposal of leukocytes by drainage, it has been shown that fewer leukocytes were mobilized in the nonligated uteri.

Phagocytosis appears to play an important part in ridding the uterus of infections produced by *Staph. aureus*. The evidence obtained fails to indicate any difference in the phagocytic activity of leukocytes from uteri in the follicular and luteal phases. It is suggested that a nonleukocytic defense mechanism may exist also in uteri during the follicular phase.

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Summario in Interlingua

Variationes del Responsa Uterin a Infection Experimental, Determinate per le Stato Hormonal del Ovarios
II. Le Mobilisation de Leucocytos e Lor Importantia in le Activitate Bactericida Uterin

Esseva interprendite un studio de varie aspectos del mobilisation de leucocytos per le uteros de conilios in le phase follicular e luteal, post infection experimental con *Escherichia coli* e *Staphylococcus aureus*. Le resultatos non indica que le differentias del numero de leucocytos circulante in le sanguine de animales in differente phases del cyclo reproductive es responsabile pro le differentia del activitates bactericida in lor uteros. Similemente, le differentias in activitate bactericida uterin non resulta de differentias in le numero del neutrophilos que es normalmente presente in le uteros durante le phases follicular e luteal ante le momento del infection. Es presentate datos que suggere que le utero in le phase follicular es capace a producer (o a liberar) un factor leucotoxic e que isto appare plus precocemente in le curso del processo infectional in uteros in le phase follicular que in uteros in le phase luteal. Le resultatos etiam suggere que il es plus difficile pro le leucocytos, post lor mobilisation ab le circulation, passar per le endometrio de uteros in le phase luteal que per illo de uteros in le phase follicular.

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Ligation ha un plus marcate effecto super le numero del leucocytos congregante in uteros in le phase follicular que in le phase luteal. A parte le elimination de leucocytos per drainage, il ha essite demonstrate que plus basse numeros de leucocytos es mobilisate in uteros non ligate.

Il pare que phagocytosis ha un rolo importante in liberar le utero de infectiones causate per *Staph. aureus*. Le datos colligite in le presente studio revela nulle differentia in le activitate phagocytic inter le leucocytos ab uteros in le phase follicular e illos ab uteros in le phase luteal. Es suggerite le possibilitate que un mechanismo de defensa non-leucocytic existe etiam in uteros durante le phase follicular.

The Inactivation of Foot-and-Mouth Disease Virus with Liquid Ethylene Oxide

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ETHYLENE OXIDE has been found to be a suitable gaseous sterilizer.[5,6] Numerous reports have been made regarding the bactericidal activity of this chemical. Although studies of the virucidal activities of this compound have been very limited, viruses responsible for influenza, Newcastle disease, western equine encephalomyelitis, and Coxsackie infection were readily inactivated with ethylene oxide.[3,11] Suspensions of foot-and-mouth disease virus (FMDV) were inactivated when exposed to ethylene oxide in the gaseous state.[1,9] It was determined, therefore, to study the inactivation of FMDV by liquid ethylene oxide (ETO) with quantitation not possible with the gaseous form of the chemical.

MATERIALS AND METHODS

Virus.—A 10 per cent tissue suspension was prepared from infected tongue epithelium of steers which had been inoculated with FMDV, type A, strain 119 (A-119). The tissue was ground in a mortar with an abrasive substance,* diluted with tryptose phosphate broth, and centrifuged at 2,000 r.p.m. for 15 minutes (900 x g) at 4 C.; the supernatant fluid was used as the virus material.

Preparation of Ethylene Oxide-Treated Suspensions.—The 10 per cent virus suspensions at 4 C. were dispensed in 19.6-ml. or 19.8-ml. amounts into flat-bottomed glass vials measuring 25 by 95 mm. An amount of ETO, chilled to 4 C., was added to each vial with a syringe, using sufficient chemical to prepare either a 1 or 2 per cent final concentration of the epoxide. Ethylene oxide is a clear, colorless liquid with a boiling point of 13 C. It is highly toxic and readily explosive at room temperature. This chemical must, therefore, be mixed with the tissue suspension at 4 C. Calibrated thermometers fitted with rubber stoppers were inserted into the vials. The vials were immersed in a constant-temperature water bath with a magnetic mixer similar to that described by Dimopoullos *et al.*[2] The vials containing 1 per cent ETO were mixed for one minute at 4 C. with this magnetic stirring device, using a Teflon-covered magnet in each container. The vials containing 2 per cent ETO were mixed for one hour at 4 C. Virus was exposed to the action of 1 per cent ETO at 37 C., and samples were withdrawn at 30 minutes, one hour, and two hours. The experiment was repeated, exposing the virus to 2 per cent ETO at the same temperature for removal at 30 minutes and one hour. A number of replicates were made, exposing the virus to 1 or 2 per cent ETO at 23 C. for 24 hours.

For infectivity tests of large volumes of inoculums, virus suspensions were dispensed in 98-ml. amounts into each of four milk dilution bottles (392-ml. total) containing Teflon-covered magnets. The bottles were also fitted with calibrated thermometers. A 2-ml. volume of ETO, chilled to 4 C., was added to each container. In all experiments, a portion of the original virus suspension was held as an untreated virus control.

Experimental Animals.—In all experiments, grade Hereford steers were used. The animals averaged about 700 lb. in weight and were approximately 1½ to 2 years old.

Infectivity Tests.—The samples of virus exposed to ETO and the control samples were each prepared in tenfold dilutions and inoculated into steers via the intradermolingual (i.d.l.) route. Two steers were used for each sample and the dosage was 0.1 ml. per site, using five sites for each dilution and four dilutions for each sample.[4] The 50 per cent infectious dose ($i.d._{50}$) titers were calculated according to the method of Reed and Muench.[8]

Another means for detecting infectivity in treated viral suspensions was the inoculation (by the i.d.l. route) of 5-ml. amounts from each vial into each of 2 steers (0.1 ml. per site).

A third method of testing for infectivity was performed with larger volumes of the 10 per cent material. Paired steers were each inoculated with 50 ml. of chemically treated virus by the intravenous (i.v.), intramuscular (i.m.), or subcutaneous (s.c). route. In addition, each animal was inoculated with 5 ml. by the i.d.l. route.

The resistance of steers showing no signs of FMDV 14 days after initial inoculation with treated virus was tested by i.d.l. challenge with dilutions of the original virus, held as frozen tissue for that purpose.

Complement-Fixation Test.—The ETO-treated and the untreated viral preparations were used as antigens in a Traub-Möhlmann complement-fixation test.[10] Serums taken after 14 days from steers that had been unaffected by the treated virus were tested to determine the presence of

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The technical assistance of Walter Harris is gratefully acknowledged.

* Alundum, produced by the Norton Co., Worcester, Mass.

complement-fixing (CF) antibody. The usual complement-fixation controls were included.

Virus-Neutralization Test.—Qualitative neutralization tests were performed in mice with serums of cattle inoculated with ETO-treated viral preparations. Two dilutions of each serum, 1:5 and 1:50, were combined with equal volumes of tissue culture-propagated FMDV-A119. The serum-virus mixtures were incubated at 37 C. for 30 minutes. Each serum-virus mixture was inoculated intraperitoneally in 0.05-ml. amounts into each of 10 suckling mice, 7 to 9 days old. Final virus concentration in the mouse inoculum was 100 $l.d._{50}$. Negative and positive serum controls were included in all tests.

RESULTS

The virus that had been treated with 1 per cent ETO for two hours at 37 C. or for 24 hours at 23 C. was noninfectious when tested in cattle (experiments 1 and 2, table 1). Virus treated with 2 per cent ETO for 30 minutes or one hour at 37 C. was also noninfectious for cattle (experiment 2, table 1). Other preparations (experiment 1, table 1) contained infective virus after chemical treatment. The challenge virus reaction in the tongues of the experimental steers showed that no measurable local resistance had developed.

When the dosage was increased to 5 ml. of a 10 per cent suspension of chemically treated virus, a different result was observed. Virus exposed to 2 per cent ETO for 30 minutes at 37 C. was infectious (experiment 3, table 1). Virus treated with 2 per cent ETO for one hour at 37 C. or for 24 hours at 23 C. was inactivated (experiment 4, table 1). Virus in contact with 1 per cent ETO for two hours at 37 C. or for 24 hours at 23 C. was also inactivated (experiments 3 and 4, table 1). The challenge virus reactions in test animals indicated that no local resistance had developed in tongues of the steers.

The 10^{-1} dilution of treated virus was noninfectious when given in 50-ml. amounts per steer by the i.v., i.m., or s.c. routes and in 5-ml. amounts by the i.d.l. route (experiment 5, table 1). Some local resistance had developed from the initial dose of inactivated virus in 4 steers, as evidenced by the titer of the challenge virus given i.d.l. The animals inoculated by the i.m. and s.c. routes demonstrated this reaction.

Complement-fixing activity of viral suspensions was unaffected by treatment with ETO (table 2). No complement-fixing antibodies were detected in the serum of cattle inoculated with treated viral suspensions during the 14 day postinoculation observation period.

TABLE 1—Infectivity Tests of Suspensions of Foot-and-Mouth Disease Virus Treated with Liquid Ethylene Oxide (ETO)

Exp. No.	Viral suspension		Viral preparation	Route of inoculation	$i.d._{50}$/ml.	Challenge $i.d._{50}$/ml.
	Dilution	Dosage				
1	10^{-1}	0.1 ml. per	Virus control	i.d.l.	7.4*	7.4
	through	site per	1% ETO, 30 min., 37 C.	i.d.l.	2.5	....**
	10^{-4}	steer per	1% ETO, 1 hr., 37 C.	i.d.l.	2.3	....**
		dilution	1% ETO, 2 hr., 37 C.	i.d.l.	0.0	7.3
		(5 sites)	1% ETO, 24 hr., 23 C.	i.d.l.	0.0	8.2
2	10^{-1}	0.1 ml. per	Virus control	i.d.l.	7.6*	7.6
	through	site per	2% ETO, 30 min., 37 C.	i.d.l.	0.0	8.0
	10^{-4}	steer per	2% ETO, 1 hr., 37 C.	i.d.l.	0.0	7.5
		dilution	1% ETO, 2 hr., 37 C.	i.d.l.	0.0	7.8
		(5 sites)	1% ETO, 24 hr., 23 C.	i.d.l.	0.0	7.8
3	10^{-1}	5 ml.	Virus control	i.d.l.	7.5*	7.5
			2% ETO, 30 min., 37 C.	i.d.l.	+	....**
			2% ETO, 1 hr., 37 C.	i.d.l.	0.0	7.9
			1% ETO, 2 hr., 37 C.	i.d.l.	0.0	8.3
			1% ETO, 24 hr., 23 C.	i.d.l.	0.0	7.6
4	10^{-1}	5 ml.	Virus control	i.d.l.	7.4*	7.4
			2% ETO, 1 hr., 37 C.	i.d.l.	0.0	7.5
			1% ETO, 2 hr., 37 C.	i.d.l.	0.0	8.6
			1% ETO, 24 hr., 23 C.	i.d.l.	0.0	7.8
			2% ETO, 24 hr., 23 C.	i.d.l.	0.0	8.1
5	10^{-1}	5 & 50 ml.	Virus control	i.d.l.	7.9*	8.5
			2% ETO, 1 hr., 37 C.	i.d.l.	0.0	8.0
			2% ETO, 1 hr., 37 C.	i.v.	0.0	8.6
			2% ETO, 1 hr., 37 C.	i.m.	0.0	6.7
			2% ETO, 1 hr., 37 C.	s.c.	0.0	7.2

0 = No visible lesions. + = positive for foot-and-mouth disease virus. i.d.l. = intradermolingual; i.v. = intravenous; i.m. = intramuscular; s.c. = subcutaneous.

* Exponent of the logarithm. ** Not done.

TABLE 2—Complement-Fixing Properties of Suspensions of Foot-and-Mouth Disease Virus Treated with Liquid Ethylene Oxide (ETO)

Antigen preparation	Antigen dilution				Antigen control	
	1:10	1:20	1:40	1:80	Anticomplement	Hemolytic
Virus control	4	4	4	0	0	4
2% ETO, 30 min.,37 C.	4	4	4	0	0	4
2% ETO, 1 hr.,37 C.	4	4	4	0	0	4
1% ETO, 2 hr., 37 C.	4	4	4	0	0	4
1% ETO, 24 hr., 23 C.	4	4	4	0	0	4

Complement control = 0; antiserum control = 4; antigen control = 4; hemolytic system = 0; 0 = complete hemolysis (no fixation): 4 = complete fixation (no hemolysis).

The ETO-treated virus did not produce neutralizing antibodies when inoculated into steers in either 5- or 50-ml. quantities, except in 2 steers given 5-ml., i.d.l. inoculations of a virus suspension treated with 1 per cent ETO for two hours at 37 C., and 1 steer given a 50-ml., i.m. inoculation of a virus suspension treated with 2 per cent ETO for one hour at 37 C.

DISCUSSION

Infectivity of FMDV in tissue suspension was destroyed by treatment with 1 per cent ETO at 37 C. for two hours, as tested in 5-ml. amounts inoculated by the i.d.l. route in steers. However, after inoculation of this preparation, neutralizing antibodies were detected in steers. Other preparations, inactivated with different concentrations of chemical and exposed for other periods of time, did not induce neutralizing antibodies when inoculated in the same volume by the same route. It may be possible to obtain a more satisfactory antigenic response and still inactivate the virus by reducing the exposure period to less than two hours at 37 C.

Virus suspensions treated with 2 per cent ETO for 30 minutes at 37 C. were noninfectious when tested in 0.1-ml. amounts of dilutions of the treated virus (experiment 2, table 1). The same preparation was infectious in 5-ml. doses of the 10^{-1} dilution as indicated (experiment 3, table 1). This difference emphasizes the pitfalls involved in testing for innocuity, since the response of the test medium may be influenced by the dosage.

This study has demonstrated that ETO can inactivate FMDV, and thus this virus is added to the list of those that may be inactivated by such means.[3,7,11] However, FMDV was inactivated in a shorter period of time than influenza, mumps,[7] Newcastle disease, and western equine encephalomyelitis viruses.[3] Complement-fixing activity of FMDV did not diminish in contrast to influenza virus similarly treated.[7]

SUMMARY

Foot-and-mouth disease virus, type A, strain 119, was treated with varying concentrations of liquid ethylene oxide. After titration in steer tongue epithelium, the viral suspension was tested for infectivity by inoculating cattle with 5-ml. amounts of a 10^{-1} dilution by the intradermolingual route and 50-ml. amounts of a 10^{-1} suspension by the intramuscular, intravenous, and subcutaneous routes. In all cases, these inoculations were compared with titrations of live virus in control animals.

Virus in contact with 1 per cent ethylene oxide for two hours at 37 C. or for 24 hours at 23 C. was noninfective when a 5-ml. dose of the 10^{-1} dilution was inoculated into steer tongues. Virus treated with 2 per cent ethylene oxide for one hour at 37 C. or for 24 hours at 23 C. was also found to be noninfective. Moreover, the virus suspension was noninfective when exposed to 2 per cent ethylene oxide for one hour at 37 C. and inoculated in 50-ml. amounts of the 10^{-1} dilution by the intramuscular, intravenous, and subcutaneous routes.

Complement-fixing activity of the virus suspensions was unaltered by treatment with ethylene oxide. However, during the 14-day postinoculation period, no complement-fixing antibodies were detected in the serums of cattle inoculated with treated viral suspensions. Neutralizing antibodies were found in the serums of 2 steers inoculated intradermolingually with 5 ml. of virus suspension exposed to 1 per cent ethylene oxide for two hours at 37 C. Another steer produced neutralizing antibodies when inoculated intramuscularly with 50 ml. of virus suspension treated with 2 per cent ethylene oxide for one hour at 37 C.

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SUMMARIO IN INTERLINGUA

Le Inactivation de Virus de Febre Aphthose con Liquide Oxydo Ethylenic

Virus de febre aphthose, typo A, racia 119, esseva tractate con varie concentrationes de liquide oxydo ethylenic. Post titration in epithelio de lingua de bove, le suspension de virus esseva testate pro su infectivitate per inocular lo in bestial, in un dosage de 5 ml de un dilution de 10^{-1} per via intradermolingual e in un dosage de 50 ml de un suspension de 10^{-1} per via intramuscular, intravenose, e subcutanee. In omne casos, iste inoculationes esseva comparate con titrationes de virus vive in animales de controlo.

Virus in contacto con 1 pro cento de oxydo de ethyleno durante duo horas a 37 C o durante 24 horas a 23 C esseva non-infective quando un dose de 5 ml del dilution de 10^{-} esseva inoculate in linguas de bove. Virus tractate con 2 pro cento de oxydo de ethyleno durante un hora a 37 C o durante 24 horas a 23 C se monstrava similemente non-infective. In plus, le suspension de virus esseva non-infective quando illo esseva exponite a 2 pro cento de oxydo de ethyleno durante un hora a 37 C e inoculate in doses de 50 ml del dilution de 10^{-1} per via intramuscular, intravenose, e subcutanee.

Le activitate de fixation de complemento del suspensiones de virus non esseva alterate per le tractamento con oxydo de ethyleno. Tamen, in le curso de un periodo de 14 dies post le inoculation, nulle anticorpore con fixation de complemento esseva detegite in le seros de bestial que habeva recipite injectiones de suspension de virus tractate con oxydo de ethyleno. Anticorpore neutralisante esseva trovate in le sero de 2 boves inoculate intradermolingualmente con 5 ml del suspension de virus exponite a 1 pro cento de oxydo de ethyleno durante 2 horas a 37 C. Un altere bove produceva anticorpore neutralisante post le inoculation intramuscular de 50 ml del suspension de virus tractate con 2 pro cento de oxydo de ethyleno durante un hora a 37 C.

The Differentiation of Subtypes (Variants) of Foot-and-Mouth Disease Virus by Serologic Methods
I. Complement-Fixation Test

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THE INCREASED interest in the use of "killed" and attenuated foot-and-mouth disease vaccines has focused attention on the subtle but important antigenic differences that exist between different samples of foot-and-mouth disease virus (FMDV) of the same immunologic type. The importance of subtype differences was demonstrated by Galloway *et al.*[3] who found that vaccinated cattle were protected to a greater degree when their immunity was challenged with the same or homologous subtype virus used in making the vaccine than against a heterologous subtype.

To facilitate study of the relationship between subtypes, it is necessary to have serologic procedures capable of detecting small antigenic differences. Traub and Möhlmann[5] showed that small antigenic differences between samples of the same immunologic type could be detected by the complement-fixation test. These findings were confirmed by Brooksby *et al.*[2] in their study of samples from the Mexican epizootic of 1946.

This report describes the further application of complement-fixation to detect subtype differences on three samples of type O FMDV. A companion paper (part II),[4] on the differentiation of subtypes (variants) of FMDV by serologic methods, describes the use of the agar-diffusion technique to detect subtype differences of the same samples.

MATERIALS AND METHODS

Virus Samples.—Three different virus samples of Vallée type O were selected for study. One sample (Ven I) was isolated from infected cattle in Venezuela in 1950. The other two samples (M 11 and CI) were isolated from infected cattle in Mexico and the Channel Island of Guernsey in 1952 and 1957, respectively. The samples had been stored as infective bovine tongue epithelium in 0.05 M phosphate buffer (pH 7.4) containing 50 per cent glycerin at 4 C.

Antigen.—Antigen was prepared from infected guinea pig pad material. The metatarsal pads were inoculated intradermally with guinea pig-adapted virus of the sample under study and collected 24 hours later. A virus was considered adapted when secondary lesions developed on forefeet and tongue of 80 to 90 per cent of the inoculated guinea pigs within 72 hours. This occurred within four to eight

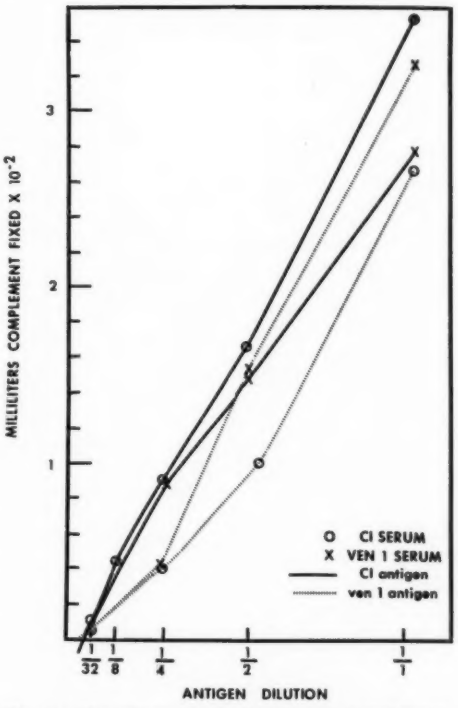

Fig. 1—The effect on the fixation of complement by the dilution of two samples of type O foot-and-mouth disease virus antigen with excesses of homologous and heterologous serum from hyperimmune guinea pigs.

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The author thanks Dr. I. A. Galloway and Dr. J. B. Brooksby, director and deputy director of the Research Institute, Pirbright, for help in providing counsel and facilities for this work. Appreciation is also expressed to Dr. R. C. Fish, chief of the USDA European Mission for Research on Foot-and-Mouth Disease, for his support of this project.

TABLE 1—Cross Complement-Fixation Reactions of Three Subtypes of Type O Foot-and-Mouth Disease Virus

Test	Antigen	Serum	Mean complement-fixation at $\frac{1}{100}$ serum dilution
III A	CI	CI	2.22 ± 0.25*
	CI	Ven I	1.14 ± 0.08
III B	CI	CI	1.58 ± 0.10
	CI	M 11	0.91 ± 0.09
III C	Ven I	Ven I	2.26 ± 0.09
	Ven I	CI	0.67 ± 0.02
III D	Ven I	Ven I	1.78 ± 0.07
	Ven I	M 11	0.12 ± .....**
III E	M 11	M 11	1.23 ± 0.03
	M 11	Ven I	0.41 ± 0.01
	M 11	CI	0.19 ± 0.01

* Readings expressed as milliliters of complement fixed × 10^{-2}. ** Insufficient readings to calculate standard deviation.

passages of these samples. A stock antigen suspension was prepared by grinding one infectious pad in sterile sand with 1 ml. of 0.05 M phosphate buffer at pH 7.4. This made approximately a 10 per cent w/v suspension of pad material. Suspensions were clarified by centrifugation at 3,000 r.p.m. for ten minutes. All suspensions were made from pads stored in glass vials at −20 C. and were used in the test within 30 minutes of preparation.

Antiserum.—Hyperimmune serums were prepared from the blood of 400- to 500-Gm. guinea pigs which were treated as follows: The metatarsal pads were infected with a stock suspension of adapted virus, followed in four weeks by an intramuscular inoculation of 1 ml. of a similar suspension made from freshly passaged guinea pig pad virus. Guinea pigs were exsanguinated ten days after the second injection, and the blood was collected in tubes coated with petrolatum. Serum was collected, pooled, and clarified by centrifugation. It was passed through a Seitz EK pad, inactivated at 56 C. for 30 minutes, and stored in 5-ml. amounts at −10 C.

Complement.—Freeze-dried guinea pig serum stored under vacuum was the source of complement.

TABLE 2—Complement-Fixation Reaction of Unknown Type O Foot-and-Mouth Disease Virus Sample Suspected of Being Either Subtype Ven I or M 11

Serum	CI		Ven I		M 11	
Antigen	CL 66	CI	CL 66	Ven I	CL 66	M 11
Serum dil.						
$\frac{1}{100}$	1.12*	1.60	>2.40	>1.92	0.60	>1.84
$\frac{1}{125}$	0.72	1.48	>2.40	>1.92	0.32	>1.84
$\frac{1}{150}$	0.56	1.40	>2.40	1.92	0.24	>1.84
$\frac{1}{200}$	0.42	1.24	2.40	1.88	0.12	1.84
$\frac{1}{250}$	0.28	1.00	2.04	1.76	0.12	0.96

* Readings expressed as milliliters of complement fixed × 10^{-2}.

The serum was obtained from the pool of serum of 100 healthy guinea pigs and dried on an Edwards centrifugal freeze drier.

Erythrocytes.—Sheep erythrocytes were obtained from a commercial source and stored in Alsever's fluid until used. For use in the test a 1.5 per cent suspension of washed sensitized cells was prepared and standardized with a colorimeter with a light filter of 525 mμ. Sheep erythrocytes were sensitized with four minimum hemolytic doses of commercially obtained amboceptor based on a 100 per cent hemolytic endpoint.

Diluent.—The diluent used throughout was veronal buffered saline at pH 7.4.

Test Procedure.—The complement-fixation technique described by Brooksby[1] for routine type determination of FMD field samples was used. This provided for a five-dilution complement series containing 0.13, 0.20, 0.30, 0.45, and 0.67 ml. of 1:25 complement dilution for each test mixture. Sufficient diluent was added to each complement mixture so that 1.0 ml. of the final preparation could be added to each tube. In addition, properly diluted antigen and serum in 0.2 and 0.4 ml. amounts, respectively, were added to the appropriate tubes. Antigen and serum controls received either 0.2 ml. or 0.4 ml. of diluent so that the final volume in each tube was 1.6 ml. After incubation at 37 C. for 30 minutes, 1.0 ml. of sensitized cells was added to each tube; the tubes were then incubated at 37 C. for another 30-minute period. The red cells were sedimented by centrifugation, and the percentage of hemolysis for each tube was determined with a colorimeter. The 50 per cent hemolysis endpoint of a test mixture was determined by probit analysis of the hemolysis over the five-dilution complement range for that mixture and expressed as milliliters of complement fixed. The specific fixation by the antigen-antibody system was the difference between the fixation of this mixture and the highest anticomplementary control.

RESULTS

Experiments were conducted to determine the effect of the dilution of antigens in the presence of excess heterologous and homologous serums on the demonstration of subtype differences. There was a linear reduction of the amount of complement fixed with decreasing concentrations of antigen, but only slight evidence of subtype preference even in the highest antigen concentration (fig. 1).

The effect of dilution of CI antigen in the presence of a homologous serum dilution series on the amount of complement fixed was plotted (fig. 2). These data indicate that FMD guinea pig antigens were not in excess when used as stock preparations. The effect of the antigen dilution was evidenced throughout the serum dilution range. The preferred test was one in

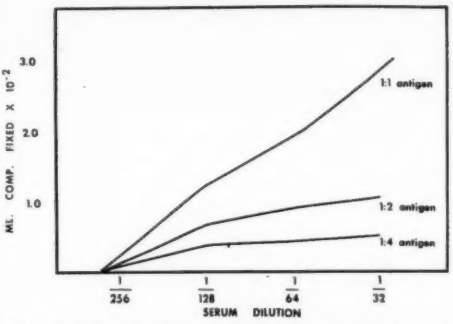

Fig. 2—The effect on the fixation of complement by the dilution of foot-and-mouth disease virus antigen in the presence of a dilution series of homologous serum from hyperimmune guinea pigs.

which a constant antigen dilution was used to titrate homologous and heterologous serums. The serum dilution range between 1:100 and 1:400 favors the serum as being the major limiting factor of fixation.

The results of the reaction of antigens with dilution series of homologous and heterologous serums is shown (fig. 3, test III, parts A, B, C, D, and E). The greatest fixation in all serum dilutions was with the homologous antigen. Slopes calculated by the method of least squares were used to correct each reading to fixation in 1:100 serum dilution. The mean fixation at this dilution is shown (table 1).

A test of an unknown sample (CL 66) suspected to be either subtype Ven I or M 11 was conducted with CL 66 guinea pig pad antigen against Ven I, M 11, and CI guinea pig hyperimmune serums with simultaneous titration of each of these serums with their homologous antigen. The results (table 2) show that CL 66 antigen had preferential fixation with Ven I serum.

DISCUSSION

The data presented confirm that antigenic differences between different samples of FMDV within a type may be demonstrated by complement fixation. It is essential, however, when comparing unknown antigens that the reaction with the homologous serum is demonstrated to be greater than any heterologous serum reaction for each virus sample when reacted with all of the serums under study. If one is reasonably sure that an unknown is one of the subtypes for which a serum has been prepared, it is possible to compare the comparative fixation of the unknown sample with the existing hyperimmune serums. This was the procedure followed in classifying CL 66.

The proposed procedure eliminates the quantitative balancing of antigens as to their comparative fixing abilities with their own serums. As long as all of the serums under study are simultaneously tested (and react) with the same antigen preparations of each of the samples, their absolute titers may differ depending on antigen concentration, but their relative titers remain constant.

The inability to demonstrate excess of antigen in the higher serum dilutions (fig. 2) could probably be eliminated by increasing the fixation time. Any gain from this procedural change, however, would be offset by the increased time for the test.

The effect of variation of antigen concentrations between tests is apparent when

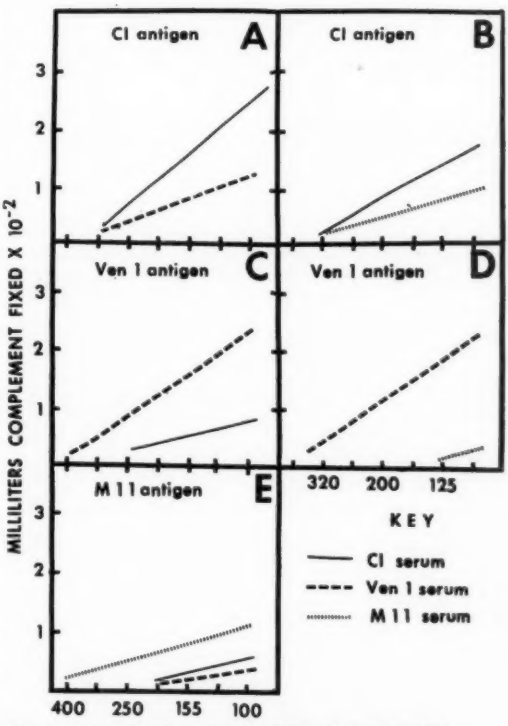

Fig. 3—The effect on the fixation of complement in hyperimmune guinea pig serum that has been titrated with homologous and heterologous subtype antigens of foot-and-mouth disease virus.

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comparing the similar reactions, CI-CI of tests III A and III B and Ven I-Ven I of tests III C and III D (table 1). The difference in antigenic content of different pad harvests is evidenced by different mean fixation figures, although the reactions are with the same serums in each test.

The suggested procedure for the subtype analysis follows the standard technique for field typing used at the FMD World Typing Center at Pirbright, England. However, the technique described in this report uses a serum-dilution series between 1:100 and 1:400, including homologous subtype serums, instead of the standard 1:16 dilution of type specific serum ordinarily used.

SUMMARY

A procedure for the quantitative determination of small antigenic differences among three subtype samples of type O foot-and-mouth disease virus is described. The greatest fixation reaction occurred when serum was titrated with its homologous antigen.

The test procedure was based on the variable complement-dilution series technique as described by Brooksby.[1] It is the standard test now used at the World Typing Center for Foot-and-Mouth Disease, Pirbright, England. In place of the standard typing serum dilution of 1/16, the procedure substitutes a dilution series of homologous and heterologous serums prepared from hyperimmune guinea pigs.

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SUMMARIO IN INTERLINGUA

Le Differentiation de Subtypos (Variantes) de Virus de Febre Aphthose per Methodos Serologic
1. Test de Fixation de Complemento

Es describite un technica pro le determination quantitative de micre differentias antigenic inter specimens de tres subtypos del type O de virus de febre aphthose. Le plus forte reaction de fixation occurreva quando le sero esseva titrate con su antigeno homologe.

Le technica es basate super illo describite per Brooksby, utilisante series de variate dilutiones de complemento. Le technica de Brooksby es currentemente le test standard usate al Centro Mundial pro le Typia de Virus de Febre Aphthose a Pirbright in Anglaterra. In loco del dilution seral standard de 1 a 16 usate a Pirbright, le presente technica se servi de un serie de dilutiones de homo- e heterologe seros ab porcos de India hyperimmun.

The Differentiation of Subtypes (Variants) of Foot-and-Mouth Disease Virus by Serologic Methods
II. Precipitin Test in Agar Gel

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A COMPANION report [3] has shown that antigenic differences can be detected by complement fixation among three different samples of type O foot-and-mouth disease virus (FMDV). For successful use of the complement-fixation test for this work, it was necessary to prepare, from hyperimmune guinea pigs, antiserum against each of the samples studied. The desire to develop a test for detection of small antigenic differences among subtypes using serum from immune cattle led to a study of the precipitin reaction in agar gel. Bodon [1] demonstrated that a precipitin reaction could be detected between FMDV antigens and immune type specific serum when the reacting substances were diffused in agar. This observation has been confirmed by Brown and Crick.[2]

The present report deals with the application of a quantitative precipitin reaction in the study of subtype differences among samples within an immunologic group.

MATERIALS AND METHODS

Virus Samples.—Three different virus samples of Vallée type O were selected for study. One sample (Ven I) was isolated from infected cattle in Venezuela in 1950. The other two samples (M 11 and CI) were isolated from infected cattle in Mexico and the Channel Island of Guernsey in 1952 and 1957, respectively. The samples had been stored at 4 C. as infective bovine tongue epithelium in 0.05 M phosphate buffer (pH 7.4) containing 50 per cent glycerin.

Antigen.—Guinea pigs weighing 400 to 500 Gm. were inoculated intradermally in the metatarsal pads with virus adapted to the guinea pig by several rapid serial passages. Vesicular fluid was collected with a Pasteur pipette 24 hours later. Since maximal antigenic concentration was desired, vesicular fluid was used undiluted in accord with the findings of Brown and Crick.[2] The vesicular fluid was allowed to clot at 4 C. for 30 minutes, clarified by centrifugation, and stored in glass vials at −20 C. until used in the test.

Bovine Antiserum.—Two steers were inoculated intradermalingually with a 10 per cent suspension of infective bovine tongue epithelium with each of the viruses under study. Blood was collected 7, 14, and 21 days after inoculation, allowed to clot at room temperature for four hours, and stored overnight at 4 C. Serum was separated from the clot by centrifugation, pooled, filtered through a Seitz EK pad, and inactivated at 56 C. for 30 minutes. It was stored in 5-ml. amounts at −10 C. until used. Serum samples were diluted in veronal buffer (pH 7.4) and stored at −20 C.

Diffusion Medium.—Bacto agar (Difco) was prepared in 5 per cent concentration in distilled water, as described by Williams and Grabar.[5] After washing in several changes of distilled water, a final concentration of 1.5 per cent was made by mixture with 0.05 M veronal buffer at pH 8.2. Merthiolate was added in a final concentration at 1:10,000 and 15-ml. amounts were poured in 10-cm. Petri dishes. Plates were held at 30 C. for four days before use in the test.

Preparation of Test.—A special cutting tool was constructed which removed 1-mm. plugs of agar 8 mm. apart. One complete serum-dilution series could be contained on one plate. Three times, at approximately 30-minute intervals, antigen was added to one well of each pair of wells and the serum dilution to the other, which made an approximate total volume of 30 μl. of material in each well. Plates were incubated at 30 C. and examined for precipitin lines at 48 hours. The endpoint of a serum was recorded as the highest serum dilution giving a definite precipitin line.

A typical test consisted of the simultaneous titration of the three samples of antiserum of each test serum with the same batch of vesicular fluid. Thus, one test consisted of nine serum titrations by one subtype antigen.

A preliminary study of the vesicular fluid showed that fresh antigen was preferable to that which had undergone several freeze-thaw cycles. Thus, antigens that had been frozen and thawed only once were used in the tests. Also, it was shown

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that plates of medium made at different times did not vary significantly from each other when used in the tests.

RESULTS

The graphs (fig. 1) show the results of the titration of three bovine serums with homologous and heterologous antigens. The highest serum titers were obtained when the serums were titrated with homologous antigen. It was also shown that appreciable titers of heterologous serums were shown with the 7- and 14-day samples, but that this cross reactivity was diminished by the end of the third week. This was especially noticeable in the heterologous serum titrations with M 11 and CI antigens.

The results of the test of an unknown sample (CL 66), suspected of being either Ven I or M 11 virus, is shown (fig. 1). The graph shows that the reaction of the three antiserums when titrated with CL 66 antigen was similar to those shown by the same serums when titrated with Ven I antigen but not to the M 11 antigen reactions with these serums. Based on these observations, the virus sample was classified as Ven I subtype.

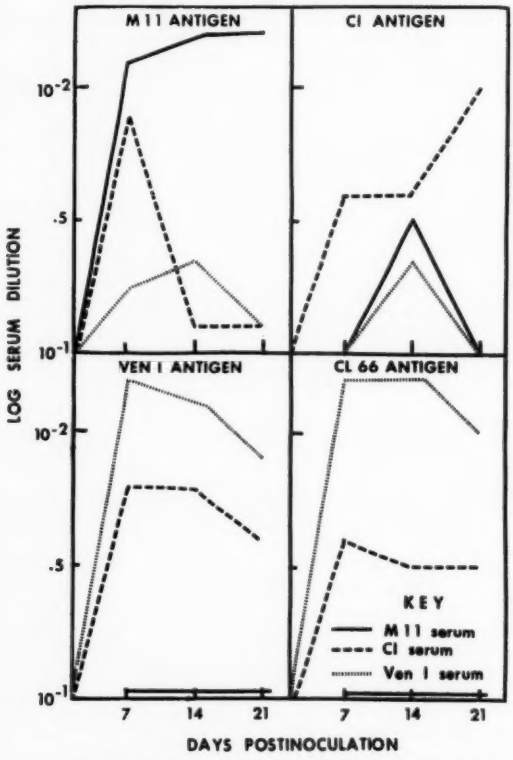

Fig. 1—The precipitin antibody response in convalescent bovine serum, after infection with three different subtypes of type O foot-and-mouth disease virus, titrated with homologous and heterologous subtype antigens.

DISCUSSION

It is clear from figure 1 that definite differences in precipitin subtype reaction exist when 21-day postinfection bovine serum is used. Comparison of serum samples drawn before this time, however, could lead to erroneous conclusions due to the cross reactive nature of early convalescent bovine serum. The preferable procedure is the one outlined, in which serum samples collected during the first three weeks postinfection are titrated by homologous and heterologous subtype antigens. In this way, the rise and fall of specific and cross reactive serum components may be measured, as well as the individual serum titers.

Skinner [4] described cross neutralization between types of FMDV with early convalescent bovine serums. Our data show that a similar cross reaction between subtypes may be demonstrated by the precipitin reaction in agar gel. It is generally accepted that the reaction shown by precipitation is a reflection of total antigen reaction and not a reaction with infective virus only. The nature of early cross reactive antibody is under study.

The precipitin reaction in agar confirms the findings reported with these same three strains in the complement-fixation study, *i.e.*, that the greatest serologic reaction will occur in the homologous reacting system and that antigenic differences between subtypes of foot-and-mouth disease may be demonstrated serologically.

SUMMARY

A precipitin test in agar gel was devised to detect antigenic differences among three subtypes of type O foot-and-mouth disease virus. The procedure involved use of homologous and heterologous bovine serum titrations by precipitation in agar gel. Definite specificity for homologous

antigen was evidenced in 21-day convalescent bovine serum. Serum during the first two weeks of convalescence, however, showed marked cross reaction with heterologous subtype antigens.

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SUMMARIO IN INTERLINGUA

Le Differentiation de Subtypos (Variantes) de Virus de Febre Aphthose per Methodos Serologic
II. Test de Precipitina in Gel de Agar

Esseva elaborate un test de precipitina in gel de agar pro deteger differentias antigenic inter tres subtypos del typo O de virus de febre aphthose. Le technica utilisa titrationes de homo- e heterologe sero bovin per precipitation in gel de agar. Un definite specificitate pro antigenó homologe esseva evidentiate in sero bovin ab animales inficite 21 dies previemente. Tamen, seros obtenite durante le duo prime septimanas del reconvalescentia manifestava un marcate reaction cruciate con antigenos heterologe.

Determination of the Optimal Age Range of Mice for Use in Experimental Studies with Foot-and-Mouth Disease Virus

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LARGE-SCALE experimental studies and diagnostic procedures requiring the use of suckling mice as detectors of foot-and-mouth disease virus (FMDV) have necessitated the mixing of young mice of different ages within experiments. Mice produced in the Plum Island Animal Disease Laboratory colony were studied to determine the age range from which equivalent response could be expected. The tests were designed to determine the age at which resistance to the lethal effect of FMDV develops.

MATERIALS AND METHODS

Mice.—Suckling mice were obtained from a self-contained colony of Rockefeller strain-H white mice maintained at the Plum Island Animal Disease Laboratory. A "litter" consisted of 10 unweaned mice of the same age, but not necessarily offspring of the same mother. Litters under test were kept in separate cages with a lactating female.

The mice of a litter of each age (in days) were inoculated with each dilution in the series. The virus was titrated in this manner at consecutive levels successively from 5 to 21 days of age. Each mouse was inoculated with 0.03 ml. of the respective dilution intraperitoneally. Each titration was repeated three times in each age series.

Virus.—The virus used was type A, strain 119 FMDV, which was present in the supernatant fluid from infected outgrowths of trypsin-dispersed bovine kidney cortical epithelium. Cellular debris was removed by centrifugation at 10,000 r.p.m. for 20 minutes in a Spinco model L centrifuge. The cell-free supernatant fluid was stored in 2-ml. volumes in sealed ampules at −40 C. until used. Virus dilutions were made in tenfold increments from 10^{-1} through 10^{-8} in isotonic tryptose-phosphate broth at pH 7.4.

The mice in the 5- to 14-day age group received virus representing the 54th passage in kidney epithelium cultured *in vitro*; the mice in the 14- to 21-day age group received virus of the 64th and 83rd passages.

Analysis of Variance.—The method of the analysis of variance was that described by Snedecor,[4] and the separation of means was the method of Duncan.[1]

In the 5- to 14-day and the 14- to 21-day groups, the mortality percentage in each litter was converted to the arc sine equivalent,[4] and the analysis of variance was performed on the converted figures. The test for variation in susceptibility of the 10- to 21-day age group by separation of means was based on the 50 per cent lethal end points of the virus as determined by the method of Reed and Muench.[3] This eliminated the dilutions of virus as a source of error in the analysis because dilution factors were considered in the end point determinations.

RESULTS

Maximal lethal effect with the strain of virus under study was found to occur between two and four days after inoculation. The primary pathologic alteration resulting from the multiplication of FMDV in suckling mice is massive muscle destruction,[2] particularly of cardiac and skeletal muscles.

TABLE 1—Analysis of Variance in Susceptibility of Rockefeller H-Strain Mice at Different Ages to the Virus of Foot-and-Mouth Disease

	Degrees freedom	Sum of squares	Mean square	F
5- TO 14-DAY-OLD GROUP				
Dilutions	7	197,228.1207	28,175.4480	326.0*
Age	9	561.4149	62.3794	<1.0
Replicates	2	8,407.1708	4,203.5854	48.7*
Dilution x age	63	5,700.3262	90.9576	1.05
Dilution x replicates	14	13,399.0004	957.0714	11.1*
Age x replicates	18	1,600.7188	88.9280	1.03
Dilution x age x replicates	126	10,877.8387	86.3320	.....
Total	239	237,774.5905	.....	.....
14- TO 21-DAY-OLD GROUP				
Dilutions	7	264,461.0535	37,780.1505	365.0*
Age	7	6,488.9370	926.9910	8.96*
Replications	2	173.8406	86.9230	<1.0
Dilution x age	49	10,324.3266	210.7005	2.04*
Dilution x replicates	14	4,358.1491	311.2964	3.01
Age x replicates	14	1,128.7214	80.6230	<1.0
Dilution x age x replicates	98	10,141.9236	103.4890	.....
Total	191	297,076.9518	.....	.....

* Denotes significance at the 1 per cent level.

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Acknowledgement is given to Mr. William Doroski for his technical assistance in this study.

The analysis of variance of the 5- to 14-day and the 14- to 21-day age groups is recorded (table 1). No differences in susceptibility of mice 5 to 14 days of age were evident, whereas the analysis of 14- to 21-day-old mice indicated that reactions to the virus varied with the ages of mice inoculated.

The analysis of the 10- to 21-day-old experimental groups (table 2) again showed that reaction variations due to ages occurred between the ages of 10 to 21 days.

The separation of the means into significant and nonsignificant differences supported the observation that mice 10 to 14 days of age tended to react similarly to the virus and that, after 14 days, resistance to virus activity increased progressively. The high titer mean of the 12-day-old mice would suggest the elimination of the fifteenth and sixteenth days from this group, but it is obvious from the data that the sharp increase in resistance occurred after the sixteenth day and increased rapidly to the 21st day. These data are presented graphically (fig. 1) and indicate that mice 21 days of age have about 20 times more resistance to the killing effect of the virus than mice 10 to 14 days of age.

There was no difference in pathogenicity of the batches of virus determined by the analysis of the 10- to 21-day age group (table 2). The batches of virus representing the 64th passage in tissue culture have been identified by RF 88, tests I and II; and the batch of virus representing the 83rd passage, by RF 107, test III.

DISCUSSION

Analysis of these observations indicates that suckling mice 5 to 14 days of age may be used when equivalent response to the

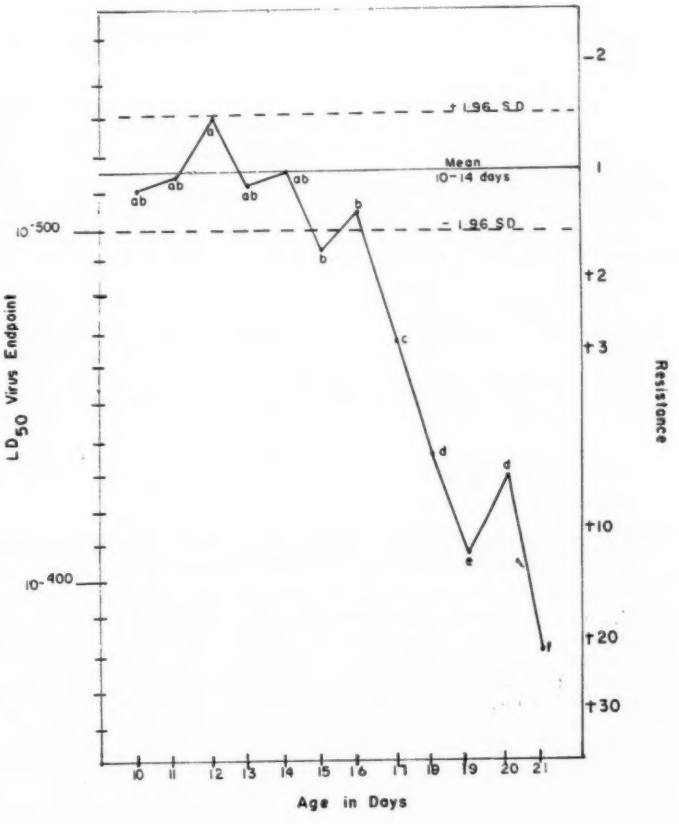

Fig. 1—Variation in susceptibility of mice 10 to 21 days of age to infection by strain 119 type A foot-and-mouth disease virus. SD = standard deviation.

TABLE 2—Variation in Susceptibility of Mice 10 to 21 Days of Age to Infection by Strain 119, Type A, Foot-and-Mouth Disease Virus

Age in days	Test Virus passage level	I RF 88 64	II RF 88 64	III RF 107 83	Mean	Stat. sig.*
10		5.00**	5.22	5.08	5.10	ab
11		5.34	4.56	5.49	5.13	ab
12		5.20	5.42	5.27	5.29	a
13		5.34	4.62	5.41	5.12	ab
14		5.34	4.67	5.41	5.14	ab
15		4.39	5.00	5.41	4.93	b
16		5.20	4.86	5.07	5.04	b
17		4.75	4.64	4.63	4.67	c
18		5.00	4.38	3.71	4.36	d
19		3.81	4.28	4.15	4.08	e
20		4.17	3.62	5.07	4.29	d
21		3.60	3.75	4.10	3.82	f
Mean		4.76	4.58	4.90	.....	..

* Statistical significance at the 5 per cent level. Mean followed by letter "a" is significantly different from those means not having "a"; those followed by "b" are significantly different from those not having "b", etc.

** Virus $l.d._{50}$ end point expressed as reciprocal of the logarithm to the base 10.

Source	Degrees of freedom	Sum	Mean	F
Age	11	7.8509	0.7137	4.38†
Tests	2	0.5983	0.2991	1.83
Residual	22	3.5836	0.1691	.....
Total	35	12.0328	.....	.....

† Significance at 1 per cent level.

virus is necessary. The difference in susceptibility in the 15- to 21-day-old group eliminates this age bracket from use in experimental work where comparative results are to be obtained. The resistance recorded must be considered a graded phenomenon.

Resistance does not imply lack of infection, but the inability of the virus to overwhelm and kill. At any age, a certain minimal amount of viable virus must be present to infect, multiply, and kill. The data indicate that a physiologic state develops in the maturing mouse which increases its resistance to overwhelming infection. The rather marked development of this resistance at the seventeenth day is of particular interest.

The host passage of virus undoubtedly has an effect on virus activity in that after serial passage in mice the virus will invade and kill more readily than virus from heterologous sources. The data illustrate that resistance is not solely a function of the genetic characteristics and dose of the virus. The major consideration is that the resistance to infection appears to be largely a function of mouse physiology.

There was no evidence from the test results that the older mice showed any latency in developing signs of illness due to virus activity.

SUMMARY

Mice produced at the Plum Island Animal Disease Laboratory (Rockefeller strain-H mice, 5 to 21 days old) were studied to determine the age ranges from which equivalent response to foot-and-mouth disease virus (strain 119, type A) could be expected.

Mice 5 to 14 days of age were found to be equally susceptible to virus activity. Resistance to the lethal effect of the virus increased rapidly after the sixteenth day.

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SUMMARIO IN INTERLINGUA

Determination del Era Optimal de Etate pro Muses Destinate al Uso in Studios Experimental con Virus de Febre Aphthose

Muses producite al Laboratorio Zoopathologic Plum Island (muses del racia Rockefeller H, de 5 a 21 dies de etate) esseva studiate pro determinar le intervallo de etate al qual un responsa equivalente a virus de febre aphthose (racia 119, typo A) poteva esser expectate.

Esseva constatate que muses de etates de 5 a 14 dies esseva equal in lor susceptibilitate al activitate del virus. Le resistentia contra le effecto lethal del virus creseceva rapidemente post le dece-sexte die.

Transfer of Immunity to Foot-and-Mouth Disease Virus from Maternal Mice to Offspring

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THE TRANSFER of immunity from mothers to their young has been studied, using a number of infectious organisms. In general, it has been observed that *in utero* transfer alone occurs in the guinea pig, the rabbit, and man; milk or colostrum transfer alone occurs in the cow and the goat; and both *in utero* and milk transfer occur in mice.[4]

The transfer of immunity to foot-and-mouth disease virus (FMDV) from immune female mice to their offspring was reported in 1952 by Skinner *et al.*[6] In the same year, Garcia-Mata *et al.*[2] observed that specific antiserum injected into pregnant mice three days before parturition protected offspring against subsequent challenge with FMDV.

The present study was designed to determine whether immunity of mice previously infected with FMDV is transferred *in utero* or through milk. Two sets of experiments were performed: the first, to repeat the work of Skinner *et al.*[6] in order to establish experimental methods and procedures; and the second, to study the route of antibody transfer.

MATERIALS AND METHODS

Virus.—The FMDV, Type A, strain 119, used in the experiments was obtained from tissue cultures of infected bovine kidney cells[1] and from infected bovine tongue epithelial tissue. Nutrient fluid from Roux flask cultures of bovine kidney cells which had been inoculated with FMDV was centrifuged (Spinco Model L centrifuge, No. 30 rotor) at 10,000 r.p.m. for 30 minutes (av. centrifugal force = 8,700 x gravity) to remove cellular debris, after which the supernatant fluid was stored in 5-ml. volumes at −40 C. in sealed ampoules.

The infected tongue epithelium was ground in a mortar with an abrasive substance,* and a 10 per cent w/v suspension was prepared with tryptose phosphate broth. This suspension was centrifuged to remove tissue debris and the abrasive substance, and the supernatant fluid containing virus was used immediately. Normal tissue-culture fluid and normal tongue epithelium used for control purposes were prepared in the same manner as the infected tissue-culture fluid and infected bovine tongue tissue. In preparing dilutions, the fluid from infected tissue cultures was considered as undiluted stock virus, while the infected 10 per cent tongue tissue suspension was considered as stock virus. All dilutions were prepared in tryptose phosphate broth (pH 7.2 to 7.4).

Mice.—Adult female mice (Rockefeller H strain) and their offspring were used in the experiments. The adult females were approximately 90 days old when they were placed with male mice, in a ratio of 3 females to 1 male. Ten days later, the females were separated from the males and placed in individual cages.

Immunization.—After the adult female mice had been with the males for ten days, they were allotted to experimental and control groups of approximately equal numbers. Each mouse in the experimental groups was inoculated intraperitoneally (i.p.) with 0.1 ml. of undiluted tissue-culture virus or 0.1 ml. of the stock 1:10 bovine tongue virus. The mice of the control groups were uninoculated or were inoculated with fluid from normal tissue cultures (0.1 ml. i.p.) or a 1:10 suspension of normal bovine tongue epithelium (0.1 ml. i.p.). Approximately 12 days later, the young were born. For convenience, dams inoculated with virus will be referred to as immune mice, while the control dams will be referred to as tissue-culture fluid controls or bovine tongue controls, depending on the material with which they were inoculated. The dams which were not inoculated will be considered as normal controls.

EXPERIMENTAL PROCEDURES AND RESULTS

PRELIMINARY EXPERIMENTS

Two experiments were performed to determine if, under the conditions described, immunity was transmitted from dams to their offspring. In the first experiment, there were two groups of pregnant mice; those in one group were inoculated with tissue-culture virus, but those in the second group were not inoculated. In the second experiment, the pregnant mice in one group were inoculated with bovine tongue virus, those in a second group were inoculated with a suspension of normal bovine tongue, and those in a third group were not inoculated. Within a few days after birth, the offspring of the mice of each group were arranged in lots of 10 mice each, the suckling mice

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* Alundum, produced by the Norton Co., Worcester, Mass.

TABLE 1—Susceptibility to Foot-and-Mouth Disease Virus of Suckling Mice from Immune and Control Mothers

Treatment of dams	Result of challenge with varying dilutions of virus								Virus end point ($l.d._{50}$/ml.)
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
EXPERIMENT 1, TISSUE CULTURE									
Virus inoculated	0/10	0/10	0/10	0/10	0/10	0/10	.....	.....	.....
Not inoculated	10/10	10/10	10/10	10/10	10/10	6/10	3/10	.....	$10^{7.70}$
EXPERIMENT 2, BOVINE TONGUE EPITHELIUM									
Virus inoculated	0/10	0/10	0/10	0/10	0/10	0/10	0/10	.....	.....
Normal tongue inoculated	.....	10/10	10/10	10/10	10/10	5/10	5/10	0/10	$10^{7.80}$
Not inoculated	.....	.....	.....	10/10	9/10	3/10	2/10	0/10	$10^{7.09}$

Numerator = number of offspring which died; denominator = number of offspring which were inoculated.

being allowed to remain with their own dams whenever possible. There were from five to seven lots for each group. The offspring were inoculated at 5 to 7 days of age with serial tenfold dilutions of the same stock virus which had been used to immunize the dams for that particular experiment. With exceptions due to the numbers of available lots, offspring in one lot from each group were inoculated with each virus dilution, using 0.05 ml. i.p. per mouse. They were observed for ten days, and $l.d._{50}$ determinations at the end of this time were made, using the method of Reed and Muench.[5]

In the second experiment, neutralization tests were performed with serums collected from the immune dams and the bovine tongue control dams, after the ten-day observation period of their offspring whose immunity had been challenged. The serums from several mice in each group were pooled, diluted 1:20 with tryptose phosphate broth, and heated at 56 C. for 30 minutes. Portions of the serums were mixed with equal volumes of tissue-culture FMDV serially diluted in tenfold increments beginning with a 1:5 dilution. After incubation in a water bath at 37 C. for 30 minutes, each mixture was inoculated into 10 offspring (one lot), 5 to 7 days old. Each mouse was given 0.05 ml. i.p. The mice were observed for ten days after inoculation.

Results.—The results of the two preliminary experiments are summarized (tables 1, 2). In the first experiment, all of the offspring of normal control dams died after inoculation with virus in dilutions of 10^{-1} through 10^{-5} (virus end point = $10^{7.70}$ $l.d._{50}$/ml.). None of the offspring of immune dams died (table 1). Similarly, in the second experiment, all of the offspring of bovine tongue control dams died after inoculation with virus diluted as much as 10^{-5} (virus end point = $10^{7.80}$ $l.d._{50}$/ml.) and none of the offspring of immune dams died (table 1).

The neutralization test performed in experiment 2 indicated that the virus-inoculated (immune) dams possessed antibodies capable of neutralizing FMDV, whereas the bovine tongue control dams did not. The titer of the virus in the presence of serum from immune dams was $10^{4.54}$ $l.d._{50}$ per milliliter contrasted with a titer of $10^{6.86}$ $l.d._{50}$ per milliliter in the presence of serum from control mice (table 2). The end point of the virus titrated without serum was also $10^{6.86}$ $l.d._{50}$ per milliliter.

FINAL EXPERIMENTS

Two experiments were performed to determine the route of immunity transfer demonstrated in the preliminary experiments. Following the procedure outlined by Larsh,[3] the offspring of immune dams were transferred immediately after birth to control dams and, at the same time, the offspring of control dams were transferred to immune dams. By this method, the roles of uterine transfer and milk transfer of immunity could be evaluated. In addition, the offspring of some of the immune and

TABLE 2—Neutralization of Foot-and-Mouth Disease Virus with Serum from Immune and Control Mother Mice

Serum source (1:40 final dilution)	Result of challenge with final dilutions of tissue-culture FMDV								Virus end point ($l.d._{50}$/ml.)
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
Mothers inoculated with FMDV-infected bovine tongue tissue	10/10	10/10	6/10	1/10	0/10	0/10	.....	.....	$10^{4.54}$
Mothers inoculated with normal bovine tongue tissue	.....	10/10	10/10	10/10	10/10	1/10	0/10	0/10	$10^{6.86}$
Virus control titration	.....	.....	.....	10/10	8/10	2/10	1/10	0/10	$10^{6.86}$

Numerator = number of offspring which died; denominator = number of offspring which were inoculated.

control dams were allowed to remain with their own dams in order to compare their response to virus challenge with that of the mice placed with foster dams. The litter size was adjusted to 10 mice in the first experiment and 8 mice in the second experiment. The first experiment involved (1) a group of immune dams and their offspring, (2) a group of immune dams nursing the offspring of normal dams, (3) a group of normal dams and their offspring, and (4) a group of normal dams nursing the offspring of immune dams. In the second experiment, there were (1) a group of immune dams and their offspring, (2) a group of immune dams nursing the offspring of tissue-culture control dams, (3) a group of tissue-culture control dams with their offspring, (4) a group of tissue-culture control dams nursing the offspring of immune dams, and (5) a group of normal dams with their offspring. The challenge virus was administered and observations were made as described previously for the preliminary experiments.

Results.—The results of two final experiments are summarized (table 3). In the first experiment, none of the suckling mice nursed by immune dams died, regardless of whether or not their natural mothers had been inoculated with virus. Of the mice born to immune dams, but nursed by normal dams, 5 of 10 mice died after challenge with the 10^{-1} dilution of virus and 9 of 10 mice inoculated with the 10^{-2} dilution died; however, no deaths occurred in mice inoculated with the 10^{-3} dilution. In contrast, mice born to normal dams and nursed by their natural mothers died after inoculation with 10^{-1} through 10^{-4} dilutions of virus (virus end point = $10^{6.65}$ l.d.$_{50}$/ml.).

In the second experiment, essentially the same results were obtained. The immunity of the offspring of immune dams was resistant to challenge, regardless of whether they were nursed by their own dams or by tissue-culture fluid control dams. Of the mice born to immune dams, but nursed by tissue-culture fluid control dams, only four deaths occurred among the 24 mice inoculated with the 10^{-1}, 10^{-2}, and 10^{-3} dilutions of challenge virus. None of these mice died after inoculation with a greater dilution. All of the offspring of tissue-culture fluid control dams nursed by their natural mothers died after inoculation with 10^{-1} through 10^{-5} dilutions of virus (virus end point = $10^{7.20}$ l.d.$_{50}$/ml.). When nursed by immune dams, however, none of the mice born of tissue-culture fluid control dams died after inoculation with FMDV. In a virus titration performed with normal mice nursed by their own normal (uninoculated) dams, all of the mice inoculated with dilutions through 10^{-4} died (virus end point = $10^{6.89}$ l.d.$_{50}$/ml.).

DISCUSSION

The results of the preliminary experiments of this study indicated that, in mice, passive transfer of immunity to offspring occurred when female mice, pregnant for about ten days, were inoculated i.p. with 0.1 ml. of FMDV containing approximately $10^{6.5}$ suckling mouse l.d.$_{50}$ per milliliter. This transfer of immunity supports the evidence of Skinner *et al.*,[6] who reported that the offspring of dams infected during pregnancy with at least 10^4 to 10^6 unweaned mouse i.d.$_{50}$ were immune to i.p. inoculation with 10^3 l.d.$_{50}$ of homologous virus.

The experiments performed to find the route of the maternal transfer of immunity

TABLE 3—Maternal Transfer of Immunity to Foot-and-Mouth Disease Virus In Utero and through Milk

Status of offspring	Status of dams nursing these offspring	Challenge dilutions of virus							
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	l.d.$_{50}$/ml.
EXPERIMENT 1									
From virus-inoculated dams	Virus inoculated	0/10	0/10	0/10	0/10	0/10	0/10	.....	.....
	Normal	5/10	9/10	0/10	.....	.....	.....	.....	.....
From normal dams	Virus inoculated	0/10	0/10	0/10	0/10	0/10	0/10	.....	.....
	Normal	.....	10/10	10/10	10/10	7/10	1/10	0/10	$10^{6.65}$
EXPERIMENT 2									
From virus-inoculated dams	Virus inoculated	0/8	0/8	0/8	0/8	0/8	.....	.....	.....
	Inoculated with tissue-culture fluid	1/8	2/8	1/8	0/8	0/8	0/8	.....	.....
From dams inoculated with normal tissue-culture fluid	Virus inoculated	0/8	0/8	0/8	0/8	0/8	0/8	.....	.....
	Inoculated with tissue-culture fluid	8/8	8/8	8/8	8/8	8/8	3/8	1/8	$10^{7.20}$
From normal dams	Normal	.....	.....	8/8	8/8	7/8	2/8	0/8	$10^{6.89}$

Numerator = number of offspring which died; denominator = number of offspring which were inoculated.

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indicated that the resistance demonstrated by offspring of immune dams had been received through the milk and *in utero* (table 3). The transfer of antibodies through the milk was suggested by the observation that offspring born to either immune or control dams were immune to challenge virus when nursed by immune dams. The *in utero* transfer is indicated by this observation that the offspring born to immune dams but nursed by control dams also resisted virus challenge. The resistance of these offspring, however, was lower than that of offspring in the other group, a fact made evident by the scattered deaths among the young mice inoculated with lower dilutions of the virus. The deaths that occurred in the latter group, however, do not necessarily mean that the immunity transfer *in utero* was lower than transfer through the milk. Since their immunity was challenged 5 to 7 days after birth, much of the antibody received *in utero* might have been eliminated before the animals were exposed to virus. This *in utero* and milk transfer of resistance from actively immunized mice supports the evidence of Garcia-Mata *et al.*,[2] who obtained similar results in work with pregnant mice immunized passively with hyperimmune serum.

The inoculation of pregnant mice with normal tissue-culture fluid or a suspension of normal bovine tongue epithelium did not stimulate a response which protected the offspring against FMDV (table 1, 3). The neutralization test performed in preliminary experiment 2, further demonstrated that mother mice inoculated with a suspension of normal bovine tongue did not produce antibodies in their serum which were capable of neutralizing FMDV. Also, the serum of mother mice inoculated with a suspension of infected bovine tongue contained neutralizing antibody. This evidence demonstrated that the resistance observed in the suckling mice was due to intra-uterine or mammary transfer of immunity from mothers or foster dams immunized with FMDV. It was not due to immunization with any substances other than virus contained in the preparations.

SUMMARY

Pregnant mice were inoculated with the virus of foot-and-mouth disease (FMDV) approximately 12 days before parturition. At birth, the offspring of immune mothers were placed with control mothers, and the offspring of control mothers were placed with immune mothers. Challenge of offspring, 5 to 7 days old, with dilutions of FMDV gave results indicating that humoral immunity was transferred *in utero* and through milk from immune mother mice to their offspring or suckling mice.

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SUMMARIO IN INTERLINGUA

Transferimento, ab Muses Matre ad le Proles, de Immunitate a Virus de Febre Aphthose

Muses pregnante recipeva inoculationes de virus de febre aphthose approximativemente 12 dies ante le parturition. Ad nato, le prole de matres immun esseva placiate con matres de controlo, le prole de matres de controlo con matres immun. Le provocation del prole al etate de inter 5 e 7 dies con dilutiones de virus de febre aphthose produceva resultatos que indica que immunitate humoral esseva transferite in utero e via le lacte ab matres immun ad lor prole o lactantes.

Anatomical and Physiologic Basis for Interpretation of the Electrocardiogram

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THE HEART is an electrical generator within the thorax. An electrocardiogram is a tracing of variations in body surface potential which has been generated by the heart and transmitted through the body volume to a surface point.[2] The form of a body surface potential is determined by (1) the nature of the electrical generator; or, more specifically, the pathways along which the wave of depolarization travels through the heart, (2) the conductive properties of the body volume, and (3) the relationship between the surface point and the heart within the thorax; or, more specifically, the relationship between the body surface point and the boundary between the depolarized and nondepolarized myocardium.

Thus, the configuration of an electrocardiogram recorded from any body surface point may be predicted when based upon the preceding three (anatomical, physiologic, and biophysical) determinants.

Volumes of descriptive literature are available on the configuration, timing, and lead systems relevant to electrocardiograms of animals. Descriptions of the constituent electrocardiographic deflections, however, give little or no thought to the genesis of the deflections. To the contrary, lead systems were designed to record canine, bovine, and equine body surface potentials nearly identical to those recorded from analogous points on the body surface of man. Frequently, interpretations were based upon resemblances to electrocardiographic patterns established as specific for particular disease conditions in man.

Thus, despite the fact that the electrical generator (heart) within the body volume may be relaying quantitatively and qualitatively different information to the body surfaces, attempts were made to interpret this information from animals by using values derived from electrocardiograms of man.

The specificity of this "descriptive electrocardiography" limits its usefulness in two ways. It renders over-extrapolation from species to species a dangerous practice. It necessitates an almost impossible accumulation of information. That is, it is impracticable to duplicate for each species (and for each age group, body conformation, and sex within each species) the comparable information accumulated by electrocardiography in man. The electrocardiographic patterns have been meticulously characterized from many body surface points for normal man and for man in numerous pathologic states.

It is the purpose of this report to present the anatomical and physiologic data which are important in determining the form of the electrocardiogram. The application of biophysical principles to such data substitutes a theoretical for an empirical analysis in the interpretation of the electrocardiograms of animals.

Although all component electrocardiographic deflections may be predicted, this report shall deal only with those generated by ventricular and interventricular septal excitation—the QRS complex.

VENTRICULAR ACTIVATION PROCESS OF THE DOG

After the excitatory impulse traverses the atrioventricular nodal conduction system, it is quickly carried by the Purkinje fibers to all areas of the endocardium and subendocardium at a velocity of 2.5 meters per second. The Purkinje system affords a method whereby almost all areas of the ventricles are stimulated and therefore contract simultaneously. Activity represented electrocardiographically occurs only when the wave of excitation traverses muscle by conduction from fiber to fiber. The electro-

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cardiograph is silent during the time when the Purkinje system alone is carrying the impulse.

The first area activated by conduction from fiber to fiber is the interventricular septum (fig. 1).[13] This activity occurs simultaneously from the left ventricular septal surface to the right, and from the right ventricular septal surface to the left. The electrocardiograph records septal activation as if only from left to right because *(a)* the left ventricular component to the septum is greater and its activation is earlier and *(b)* the electrocardiograph determines the resultant of all of the simultaneous activation fronts, giving a magnitude proportional to the uncancelled muscle mass activated. This event requires less than 0.01 second for completion.

After interventricular septal activation, the impulse spreads by fiber-to-fiber conduction through the ventricular myocardium from the subendocardial terminations of the Purkinje system to the epicardial surfaces.[4,12] This activation occurs simultaneously through both ventricles. However, the peripheral electrocardiogram, which represents the resultant of the simultaneous activities of both ventricles, is recorded as if the front were moving only from the left ventricular endocardium to left ventricular epicardium because the left ventricle is four to six times as thick as the right. This activity requires 0.025 to 0.035 second for completion.

Finally, the ventricular activation process is terminated when the base of the heart is activated in an apicobasilar direction.[4,12]

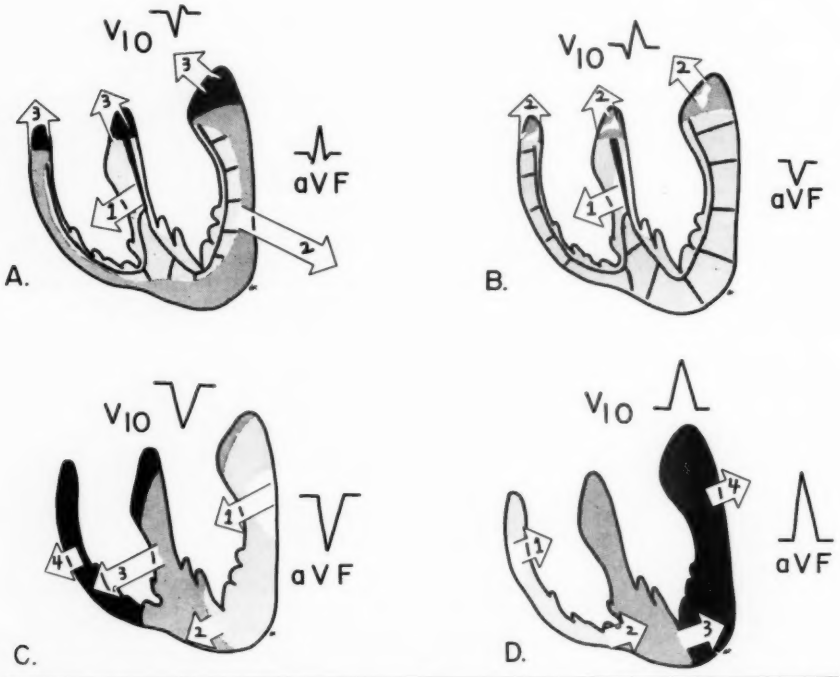

Fig. 1—Left lateral view of sagittal sections of hearts, showing right ventricle to the left of the interventricular septum, and the septum to the left of the left ventricle. Lead V_{10} is above the base of each heart, and lead aVF lies to the right of each heart. The distribution of the Purkinje system is shown in black lines. The order of ventricular activation is shown by shading, with earliest activated areas lightest, and latest activated areas darkest. Major fronts of activation are represented by numbered arrows. (A) Normal canine heart, (B) Normal ovine heart, (C) Either canine or ovine heart with fiber-to-fiber activation from left ventricular-epicardial premature beat, and (D) Same as C, only for right ventricular-epicardial premature beat.

This fiber-to-fiber spread requires 0.01 second.

Thus, although many complex and algebraically canceled fronts of activation comprise the ventricular activation process, temporarily only three major fronts of activation (dipoles) may be described in the activation of the ventricles (fig. 2).[8,9] These dipoles may be expressed as vector quantities having magnitude proportional to the muscle mass activated and direction according to the pathway through the heart. Vector one represents activation of the interventricular septum from left to right. Vector two represents activation of the ventricular myocardium from the left ventricular endocardium to the left ventricular epicardium. Vector three represents activation of the septal and ventricular bases in an apico-basilar direction. It must be emphasized that, for ease of understanding, a single vector of appropriate magnitude and direction is used to represent all of the complex electrical forces generated by the excitation of given volumes of muscle in given directions at a single instant.

BIOPHYSICS OF ELECTROCARDIOGRAPHIC AND DIPOLE AXES

Biophysical principles applied to electrocardiography[1,16] are extremely complex and numerous; however, those salient to this report may be simply stated as:

1) When the wave of electrical negativity (the front, or dipole, of the ventricular activation process) travels toward the exploring (positive) electrode of the galvanometer, a positive deflection is recorded. When the wave travels from the positive pole toward the negative pole, a negative deflection is recorded. When the dipole is directed at right angles to both poles of the galvanometer, no deflection occurs. The latter fact emphasizes the need for multiple leads.

2) For more accurate approximation (fig. 3), the amplitude of the galvanometer deflection is *(a)* directly proportional to the uncanceled current dipole, *(b)* inversely proportional to the square of the distance between the dipole and the galvanometer leads, and *(c)* directly proportional to, and of the same sign as, the cosine of the angle formed between the lead axis and the vector representing the dipole.

With knowledge of *(a)* these elementary biophysical applications, *(b)* the location of the galvanometer electrodes on the torso and the poles to which they lead, and *(c)* the sequence of ventricular activation, one need only know where the heart lies within the thorax to determine qualitatively what electrocardiogram configuration to expect in a given lead.[8,11]

Position of the Heart Within the Thorax.—In most mammals, except primates, the heart is located as illustrated (fig. 4).[6,14,17] Notice particularly that the right ventricle lies cephalad and dextrad to the interventricular septum. Also notice that the interventricular septum forms a plane deviated from the median sagittal plane by angle Ω (45 degrees), and that it is tipped with its dorsal border cephalad to the ventral border forming angle ϕ (45 degrees) with the ventral plane.

The determinates proposed earlier have now been described. To arrive at a qualitative analysis of the ventricular activation process by correlating the preceding information, it is necessary to decide which electrocardiographic leads are necessary for complete registration of the electrical forces generated by the heart.

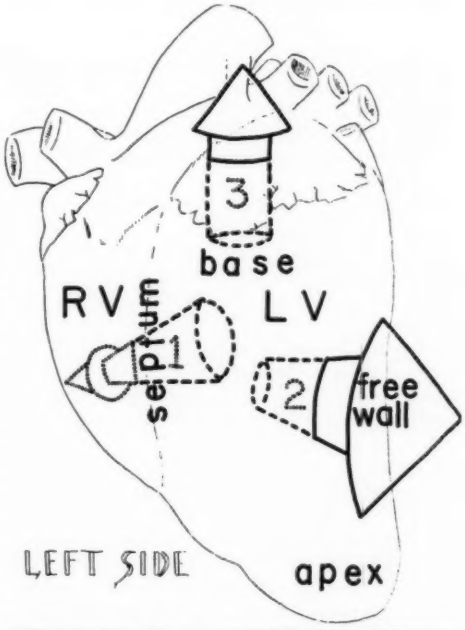

Fig. 2—Heart of dog viewed from left lateral aspect, showing three resultant vectors representing major fronts of activation.

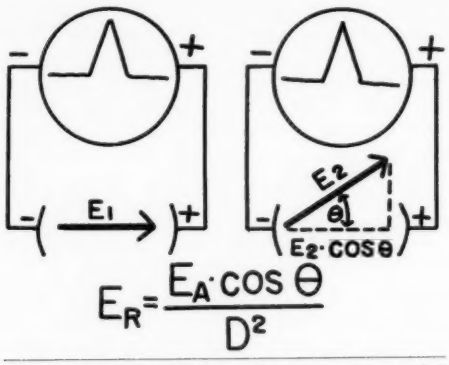

Fig. 3—Biophysical relationship between recorded voltage (E_R), actual voltage (E_A), distance between dipole and electrocardiographic leads (D), and angle formed between vector representing actual dipole and the projection of the vector on the lead axis (angle $\ominus$). So long as the angle is less than 90 degrees (i.e., the vector is directed from left to right), the cosine of the angle will be positive and the recorded voltage will be positive. As the angle exceeds 90 degrees, the cosine becomes negative and the recorded voltage is negative. The more perpendicular the vector is to the lead axis, the smaller the cosine will be, and the smaller the recorded voltage will be. Positive and negative poles of the galvanometer are marked accordingly. Deflections caused by the voltages between the electrodes are drawn in circles above.

Any and all vector forces representing the resultant of many instantaneous and canceling waves of activation may have magnitude along one or a combination of three axes (fig. 5): dorsoventral (Z axis), cephalocaudal (Y axis), or sinistrodextral (X axis). To determine the relative magnitude and direction of the vector in space, one must have an electrocardiogram which determines activity along each of the three axes: that is, an electrocardiographic lead which will have its positive and negative pole at either extreme of the X, Y, or Z axis. Applying the cosine law to the projection of the vector along all three axes, the magnitude and direction of the vector in space may be determined. The triaxial reference system recommended here (fig. 5) has its origin within the heart. Each axis extending from the heart corresponds to an anatomical body axis and is determined by an electrocardiographic lead. Thus, for this system, three electrocardiograms are necessary to obtain all of the information projected to these three axes. This concept is theoretical because the instantaneous single dipole representation is an over-simplification of the complex ventricular activation process. Furthermore, because certain inhomogeneities exist within the torso as a volume conductor, the relationship cited between the body surface potential and the boundary between the depolarized and nondepolarized myocardium does not result in more than a semiquantitative estimate of the electrocardiogram.

Lead I (positive pole on the left thoracic limb, negative pole on the right thoracic limb) measures differences of potential between the two thoracic limbs and, therefore, determines X axis forces. Leftward-directed forces will cause a positive deflection in lead I. Lead aVF (unipolar lead with the positive electrode placed on the left pelvic limb and the other electrode at the Wilson central terminal), lead II (positive pole on the left pelvic limb, negative pole on the right thoracic limb), and lead III (positive pole at the left pelvic limb, negative pole at the left thoracic limb) measure differences of potential between the extreme caudal and cephalic aspects of the animal and, therefore, determine Y axis forces. Caudally directed forces will result in a positive deflection in these leads. Lead V_{10} (unipolar lead with the positive electrode placed on the dorsal spinous process of the seventh thoracic vertebra and the negative electrode at the Wilson central terminal) measures differences of potential between the dorsal point and the "electrical" center of the heart; therefore, it determines the Z axis forces. Forces directed dorsally will result in a positive deflection in this lead.

In assigning a spatially oriented vector for each of the dipoles of the ventricular activation process, it is possible to anticipate qualitatively what the electrocardiograph configuration will be in leads I, aVF, and V_{10} and for all other peripheral electrocardiograms recorded from known axes.[11]

Vector one (the septal vector) is directed equally cephalad and ventrad, and slightly dextrad. It has small magnitude because it represents activation of a small muscle mass. One would expect a small negative deflection in lead aVF (the forces going away from the positive pole of the lead), a small negative deflection in V_{10} (the forces being directed ventrally away from the positive pole of this lead), and a slightly negative deflection in lead I (the forces being directed away from the positive left thoracic limb electrode of the lead).

Vector two, the resultant of ventricular free-wall activation, is directed almost strictly caudally with only slight ventral and sinistral orientation. It has great magnitude because it represents activation of the greatest muscle mass. As a result, a larger positive deflection occurs in lead aVF (the forces traveling toward the positive pole of the lead), a small negative deflection in V_{10} (the forces still traveling slightly away from the positive pole of the lead), and a slightly positive deflection in lead I (the forces directed sinistrally toward the positive pole of that lead).

Vector three, representing apicobasilar activation of the ventricular and septal bases, is directed dorsally and cephalically, with litle sinistral-dextral orientation. This vector, representing activation of only a small muscle mass, will be of small magnitude. Thus, there occurs a positive deflection in lead V_{10} (the forces being directed dorsally toward the positive electrode of the lead), a negative deflection in lead aVF (the forces being directed cephalically away from the positive electrode of this lead), and little or no deflection at this time in lead I because none of the vector forces are directed either sinistrally or dextrally.

Hence, it becomes obvious how a simple analysis of the ventricular activation process may enable one to anticipate the ventricular complex of the electrocardiogram-determining forces along a known axis. When the ventricular activation process, the relationship between the cardiac dipoles and the torso, and the relationship between the lead axis and the torso are known, the accurate construction of the three-reference electrocardiogram is easily accomplished. In practice, the process is simply reversed. The electrocardiogram configuration in the three leads and the relationship between the lead axes and the axes of the heart are known. The problem becomes the estimation of the ventricular activation process.

In locating the spatial orientation of any given instantaneous vector from the three-reference electrocardiograms (X, Y, Z), care must be exercised to measure the same time on each electrocardiogram which corresponds to the time of the instantaneous spatial vector being derived (fig. 6).

Examples

Suppose leads I, aVF, and V_{10} have large positive deflections. From this, it is known that the waves of excitation are traveling so as to generate a spatially oriented vector directed caudally, dorsally, and sinistrally.[7] There appears to be no activation of the interventricular septum from left to right which would have generated an initial vector directed ventrally and cephalically. From this, one may conclude that there is either a left bundle-branch system block, or that the beat studied was ectopic and originated from the right ventricle and spread

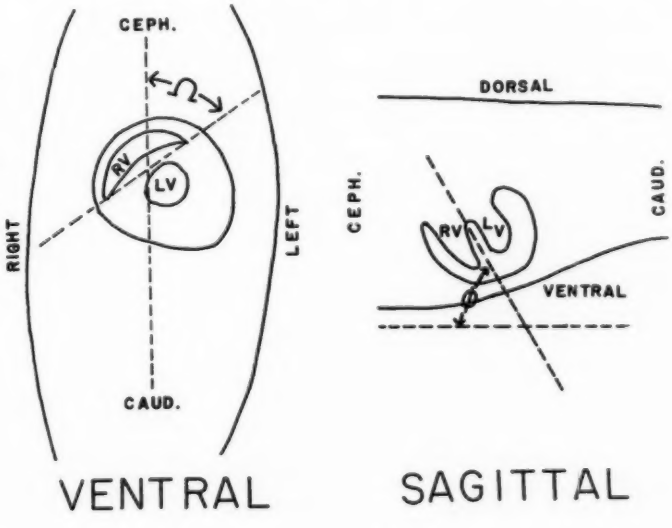

Fig. 4—Schematic drawings of ventral and sagittal cross-sections through thoraces of normal dogs. Relationships between the plane of the interventricular septum and the median sagittal plane and ventral plane of the body are described by angles Ω and Ø.

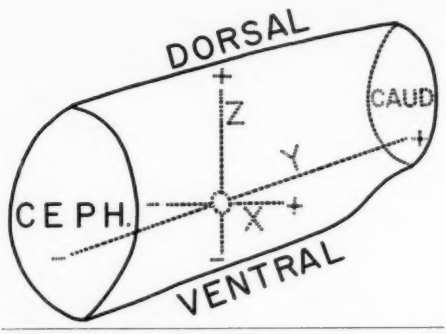

Fig. 5—Left dorsocephalic view of canine torso, schematized to show three lead axes (X, Y, and Z) with their polarity.

via muscle fiber to muscle fiber conduction, first through the right ventricle and then through the left (fig. 1d). In addition, one would expect large amplitude in lead aVF, since all of the heart muscle mass is activated in an uncanceled manner toward the positive electrode of the lead.

The same approach may be applied to premature beats originating from left ventricular foci (fig. 1e). The complexes in leads aVF and V_{10} for this case, however, would be all negative and of large amplitude, since all of the forces are directed away from the ectopic focus and therefore away from the positive poles of leads V_{10} and aVF.

If an animal has marked right ventricular hypertrophy, the summation of left and right ventricular activation could produce a vector directed cephalically instead of caudally. Thus, lead aVF would have, initially, the negative component of caudal to cephalic septal activation and, finally, a component of right ventricular activation also directed cephalically.

The scope of this report does not include electrocardiographic characterization of all of the many possible conditions of aberrant intraventricular conduction. The value of the report should lie in the presentation of an anatomical and biophysical method for the estimation of any condition of ventricular conduction, given electrocardiogram-determining forces along the three body axes and knowing the relationship of the cardiac structures to these leads.

Two limitations must be considered. The first stems from the fact that the absolute center of any given dipole may not be located by conventional electrocardiograms. From the peripheral electrocardiogram, one may determine the time sequence of successive resultant vectors. However, it is equivocal whether the initial cephaloventral vector represents activation of the interventricular septum in that direction, the ventricular free-wall from epicardium to endocardium, or the right ventricular free-wall from endocardium to epicardium. In all cases, the forces would be represented by identical vectors.

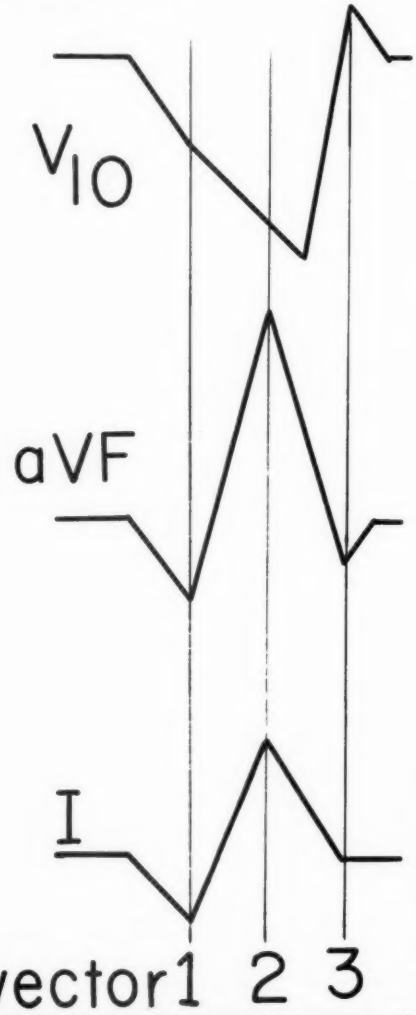

Fig. 6—Electrocardiographic leads I, aVF, and V_{10}, reconstructed on simultaneous coordinates to show corresponding time points for each of the three major vectors.

Some assistance in determining a more precise location of the dipole within the heart is obtained by recording thoracic leads from points close to the heart. Interpretation of such electrocardiograms is particularly difficult because the position of the heart within the thorax compared to single external landmarks on the thorax is extremely variable. In addition, because of greater lead-proximity to the heart, animals with narrow thoraces will have larger deflections than will those with more rounded thoraces; so, unless the thoracic electrocardiograms of each body build are meticulously characterized, the interpreter will not know whether a particular recording showing a high position deflection at the left fifth intercostal space is due to a narrow thorax, left ventricular hypertrophy, or aberrant ventricular activation.

The second limitation is extremely important in ruminant and probably in equine electrocardiography. Ventricular excitation in these two groups of animals [10, 15] occurs in a different time-order than for the dog, cat, man, pig, and most animals with simple stomachs. Because of the more complete penetration of the Purkinje system into the epicardium of the ruminant and equine (fig. 1b), all areas of the ventricles except the base are excited simultaneously soon after interventricular septal excitation.[3] Since the electrocardiograph measures only muscle fiber to muscle fiber activation, and since the only fiber-to-fiber spread of activation is, initially, across the septum in a caudal to cephalic direction and, finally, through the base in an apicobasilar direction only, two vectors represent the ruminant ventricular activation. The middle, high-magnitude, ventrocaudal vector of left ventricular free-wall activation is absent. Although the time-order of ventricular activation through the horse heart has not been thoroughly studied, experiments suggest the same to be true with this animal as well.

The authors wish to emphasize that it is possible to approach interpretation of the ruminant and equine electrocardiogram in the same way as in the dog, if the three conditions of the premise are fulfilled for the given species. Failure in the applicability of this technique in analyzing the peripheral electrocardiogram of any animal results from lack of anatomical information or knowledge of the biophysical concepts.

Over-extrapolating patterns of the human electrocardiogram in veterinary electrocardiography is a poor substitute for the above knowledge. Although the human ventricular activation process is apparently identical to that of the dog, the human heart within its thorax lies in such fashion that identically named electrocardiograph leads determine forces directed along entirely different axes. To say that the canine heart is "like the human heart except for being electrically vertical" is a totally incorrect over-simplification. It is true that canine leads II and III are normally of equal amplitudes, and that this relationship usually indicates a "vertical heart" in man. However, the canine leads II and III determine forces along the same axis as does lead V_6 in man. Leads aVR and aVL in man determine forces directed along the X axis; in the dog, both leads determine forces along the Y axis, since both leads are more cephalad to the heart than either is to the right or to the left of the heart. This fact is well demonstrated anatomically.[17] Thus, interpretation of electrocardiograms of animals based on patterns extrapolated from electrocardiograms of man may, without knowledge of lead axis-dipole axis orientation differences, result in errors of interpretation. It appears coincidental that leads II and III in the dog look like leads II and III in man.

Summary and Conclusions

By recording an electrocardiogram from each of the three axes to determine all of the cardiac dipoles, an objective approximation of the normalcy or aberrancy of ventricular activation may be obtained from the following anatomical and physiologic considerations:

1) The normal time-order of excitation of the cardiac structures.

2) The relationship between the axes of the cardiac dipoles and the electrocardiographic lead axes.

3) The orientation of the cardiac structures to the torso.

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SUMMARIO IN INTERLINGUA

Le Base Anatomic e Physiologic pro le Interpretation del Electrocardiogramma

Per registrar un electrocardiogramma ab cata un del tres axes pro determinar omne le dipolos cardiac, un approximation objective del normalitate o aberrantia in le activation ventricular pote esser obtenite ab le sequente considerationes anatomic e physiologic:

1. Le normal ordine temporal del excitation del structuras cardiac.
2. Le relation inter le axes del dipolos cardiac e le axes del derivationes electrocardiographic.
3. Le orientation del structuras cardiac al torso.

Scientific Notes

Demonstration of Siliceous Deposits in the Kidneys of the Guinea Pig

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Ottawa, Ontario

THE OCCURRENCE of siliceous urolithiasis in range steers of western Canada and the United States has been reported by Swingle,[6] Connell, et al.,[1] and Whiting et al.[7] A description by Forman et al.[2] indicates that siliceous calculi are composed primarily of hydrated, noncrystalline silicon dioxide and organic matter.

Work in this laboratory[4] has shown that orally administered particulate silica with an average particle size of 250Å is not excreted in the urine as such, but is first depolymerized to the soluble state. It was also noticed that soluble silica in urine did not polymerize unless the concentration exceeded 18 mg./100 ml. This indicates that the nucleus of a siliceous calculus is probably formed at some point in the urinary tract.

This study presents evidence to show that, following the administration of relatively large amounts of soluble silica to guinea pigs, the formation of siliceous deposits can occur in the renal tubules.

MATERIALS and METHODS

A solution of sodium metasilicate ($Na_2SiO_3 \cdot 5H_2O$) was prepared for intraperitoneal administration. Immediately before use, the solution was adjusted to pH 5 by rapidly adding 3N hydrochloric acid. An organic silicate, tetraethyl orthosilicate, was used for oral administration. The 15-ml. intraperitoneal dose contained 20 mg. of silicon dioxide per milliliter; the oral dose of 2 ml. of tetraethyl orthosilicate solution contained 253.8 mg. of silicon dioxide per milliliter.

Adult guinea pigs, raised on a low-silica diet,[4] were killed 24 hours after the administration of silica. Preliminary tests showed that, with the levels of silica administered, many animals did not survive longer than 48 hours. At necropsy, the kidneys were pale, greatly enlarged, and had roughened surfaces; when sectioned, they were shown to have tubular deposits similar to those seen in the present experiment.

Serial sections of kidney, 10 μ in thickness, were made. Figures 1, 2, and 3 show sections taken from an adult male guinea pig that were given silica intraperitoneally. The first section (fig. 1) was stained with hematoxylin and eosin and examined by transmitted light. The silica deposits were seen in the kidney tubules as lightly stained, homogeneous-appearing masses. An adjacent section (fig. 2) was ashed in a muffle furnace at 600 C. for one hour after removal of paraffin. The slides were always ashed in covered Petri dishes in order to avoid surface contamination. The silica deposits seen in figure 1 are shown in figure 2 as they appear after ashing when viewed by reflected dark-field illumination. An outline of the cell structure of the kidney is readily visible in this section.

In figure 3, the same section is viewed by reflected dark-field illumination after a 30-minute immersion in 50 per cent (v/v) hydrochloric acid. This treatment completely removed the tissue salts that were left after ashing and, in kidney sections from control animals, removed all visible background. The silica deposits (fig. 3) were optically isotropic when viewed by polarized light. The ashed section was then treated with 0.5 per cent hydrofluoric acid for 60 seconds, dried, and examined. The first hydrofluoric acid treatment severely attacked the particles shown; one or two additional 60-second exposures to hydrofluoric acid completely removed them.

Figures 4 and 5 are photomicrographs of kidney sections that were ashed but not treated with acid. These were taken from 2 guinea pigs that were each given silica orally. It was again noticed that the deposits were not attacked by hydrochloric acid, but were readily removed by treatment with dilute hydrofluoric acid.

RESULTS and DISCUSSION

These results indicate that, when guinea pigs are given relatively large quantities of soluble silica, siliceous deposits may form in the renal tubules.

Previous work with labeled silicon[5] has shown that, when a freshly prepared sol is orally administered to guinea pigs, the silicon is rapidly excreted in the urine. It was also noticed that, when labeled silicon was given in the form of sodium silicate

From the Animal Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Ont. Contribution No. 11.

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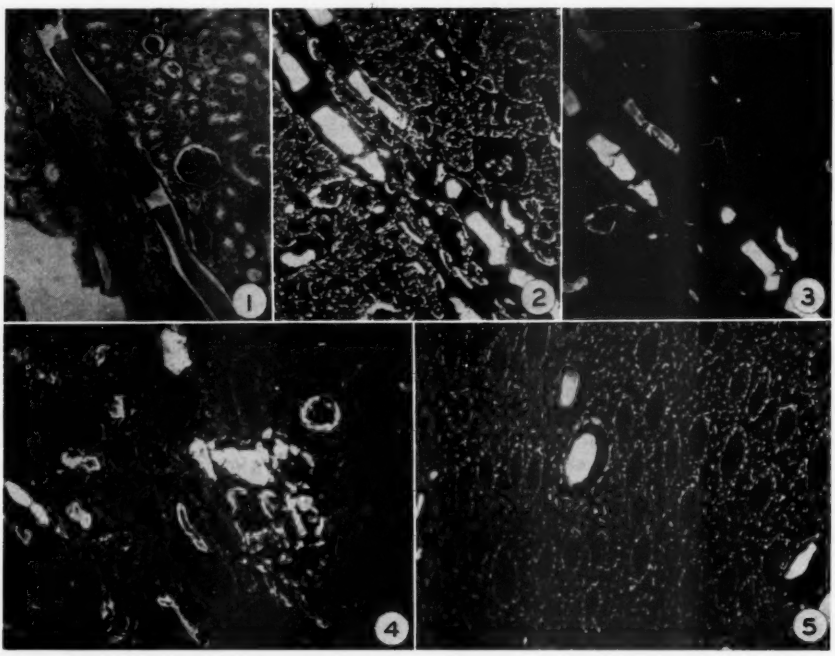

Fig. 1—Silica deposits in kidney of guinea pig given silica sol intraperitoneally. H & E stain; x 88.

Fig. 2—Section of kidney adjacent to that shown in figure 1. After ashing, same silica deposits are shown, viewed by reflected dark-field illumination. A microscopic outline of the kidney structure remains after ashing. x 88.

Fig. 3—Section shown in figure 2 following a 30-minute exposure to 50 per cent hydrochloric acid. The kidney ash has been completely removed but the silica deposits are not affected. Reflected dark field; x 88.

Fig. 4—Silica deposits in ashed sections of kidney from guinea pig given an oral dose of tetraethyl orthosilicate. Reflected dark field; x 88.

Fig. 5—Silica deposits in an ashed section of kidney from guinea pig given tetraethyl orthosilicate orally. Reflected dark field; x 88.

instead of a sol, the radioactivity in the kidney was several times greater than that present in other tissues.

It therefore appears likely that, when soluble silica is administered to guinea pigs, the silica concentration in the glomerular filtrate and urine increases until polymerization occurs and silica deposits are formed. The possibility may be considered that a similar process plays a role in the development of silica calculi.[3]

SUMMARY

Evidence showed that siliceous deposits form in the kidneys of guinea pigs given large doses of soluble silica orally or intraperitoneally. The amount of soluble silica administered was considerably greater than animals would normally ingest on a forage diet, and it cannot be concluded that silica calculi in range steers form from 'nuclei' of polymerized silica similar to those shown in the guinea pig kidney. Nevertheless, this work indicates that insoluble deposits of silica may form in kidney tubules if large quantities of soluble silica are administered to an animal.

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SUMMARIO IN INTERLINGUA

Le Demonstration de Depositos Silicee in le Renes de Porcos de India

Depositos silicee esseva demonstrate in le renes de porcos de India tractate con grande doses de silice per via oral o intraperitoneal. Le quantitates de solubile silice administrate esseva considerabilemente plus grande que lo que animales ingere normalmente in un dieta de forrage, e le conclusion non es justificate que le calculos de silice incontrate in boves de pastura es formate ab nucleos de polymerisate silice del genere demonstrate in le renes de porcos de India. Nonobstante, iste investigation demonstra que le formation de insolubile depositos de silice es possibile in le tubulos renal, si satis grande quantitates de silice solubile es administrate a un animal.

Meetings • • •

• • • American Veterinary Medical Association, 97th Annual Meeting. Denver, Aug. 15-18, 1960. H. E. Kingman, Jr., 600 S. Michigan Ave., Chicago 5, Ill., executive secretary.

• • • First International Congress of Histochemistry and Cytochemistry. Paris, Aug. 28-Sept. 3, 1960. Dr. R. Wegmann, Laboratoire d'Histochimie, Faculté de Médecine, 54, rue des Saints-Peres, Paris 6e, France, secretary general.

• • • Tenth International Congress of Cell Biology. Paris, Sept. 4-9, 1960. Prof. M. Chevremont, 20, rue de Pitteurs, Liege, Belgium.

• • • European Conference on Electron Microscopy. Delft, Netherlands, Aug. 29-Sept. 3, 1960. Prof. J. B. LePoole, Mijnbouwplein 11, Delft, Netherlands.

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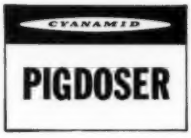

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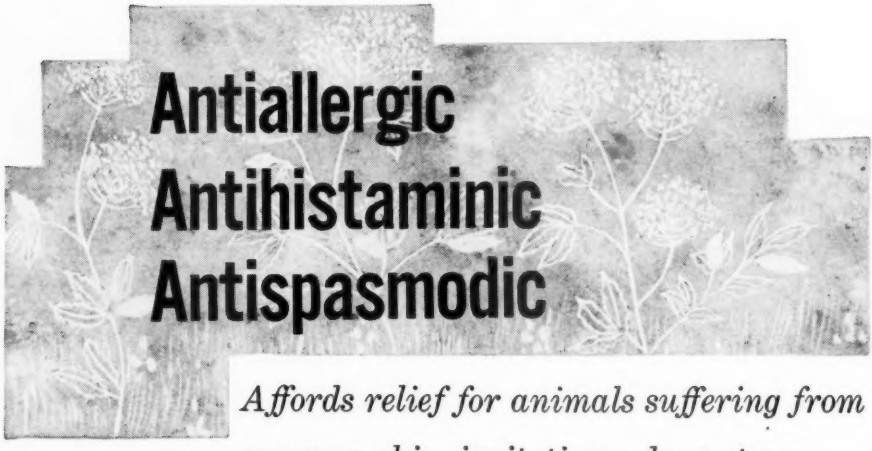

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American Journal of Veterinary Research

Volume XXI September, 1960 Number 84

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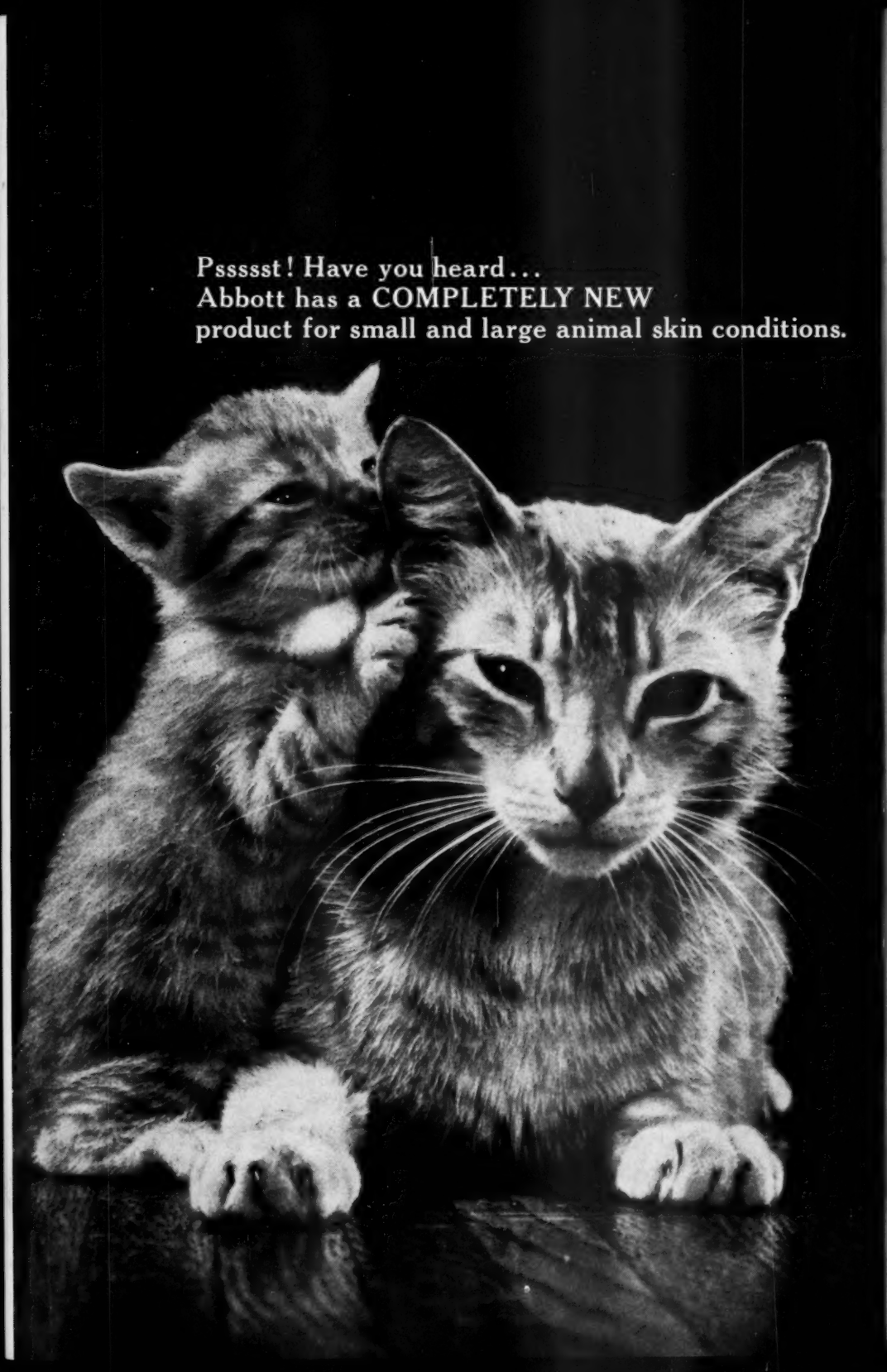
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